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Effect of water stress and mixed types of the genus Arbuscular Mycorrhizal Fungi from cocoa roots on spore propagation and root colonization used corn as the host

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Abstract

Biofertilizer with Arbuscular Mycorrhizal Fungi (AMF) as the inoculants is needed in organic cocoa cultivation on smallholder plantations in Indonesia. AMF biofertilizer requires a sufficient number of spore inoculants, so it is necessary to multiply isolated spores. Research objective was determining the effect of water stress and mixed types of AMF genus from cocoa roots on spore propagation and root colonization used corn as the host plant. The study used a 2-factors randomized block design and 3 replications. The first factor was water stress consisting of 3 levels (without water stress as a control, soil moisture content 100% of field capacity; light water stress, soil moisture content 70% of field capacity; and heavy water stress, soil moisture content 40% of field capacity), while the second factor was mixed types of AMF genus also consisting of 3 levels (inoculant of genus *Glomus* only, mixed inoculants of the genus *Glomus* + *Acaulospora*, and mixed inoculants of the genus *Glomus* + *Scutelospora*. Result of research showed, the interaction of water stress and mixed types of the AMF genus had no significantly affect on the number of spores reproduced, root colonization and host plant growth. The best way of propagation of AMF spores was by heavy water stress treatment with a soil moisture content of 40% of field capacity producing 6,713.40 spores or an increase of 13,326.82%. Mixed inoculants of the genus *Glomus* + *Scutelospora* gave the highest number of spores after propagation (6,263.40 pieces) or an increase of 12,427.77%.

Keywords: Arbuscular Mycorrhizal Fungi (AMF); Cocoa; Propagation; Spore; Water stress

1. Introduction

Cocoa (*Theobrema cacao* L.) is very important plantation commodity for Indonesia, and Indonesia itself occupies the third largest position as a world cocoa producer after Ivory Coast and Ghana [1]. Cocoa is used as raw material for agro-industry, especially the food and beverage industry as well as cosmetics and pharmaceuticals. The processed cocoa fruit, called chocolate, has very good health benefits such as reducing the risk of heart disease and increasing relaxation and memory [2-3].

The increasing demand of organic cocoa from buyers has caused many producers switch to organic cocoa cultivation. The main obstacle in producing organic cocoa on smallholder plantations is that the average yield per hectare is still very low, only ranging from 465 to 720 kg of dry beans/ha/year, even though the potential production can reach 2,000 kg of dry beans/ha/year. This happened because farmers have not implemented the 5 correct fertilization methods, i.e., the right type, dose, time, way, and target, and have not applied good agricultural practices. The reason is that they have difficulty getting organic fertilizer as recommended amount for cocoa, which is 15 tons per hectare [1], and also requires a lot of labor in transporting and fertilizing. With this cultivation method, cocoa plants lack of nutrients and cause their production is low. Use of Arbuscular Mycorrhizal Fungi (AMF) that is the fungus associated with roots and functions to help plants absorb nutrients and water as biofertilizer, is an alternatively that can be done. AMF biofertilizer is effective in small amounts, don't need a lot like organic fertilizers [4-6], so farmers can easily apply them. Rai et al. [7] found that

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indigenous endomycorrhizal biofertilizer improved the nutrient level, the relative water content of the leaves, and the photosynthesis process so increased the yield and quality of the fruits in organic snake fruits.

The production of AMF biofertilizer requires adequate number of spore inoculants, so it is necessary to multiply the isolated spores [8-10]. One method for propagation of AMF spores is by giving water stress treatment [11]. According to Diputra et al. [12] application of water stress with a soil moisture content of 40% of field capacity gave the highest number of spores in the propagation of indigenous endomycorrhizal spores taken from snake fruit roots. Wedagama et al. [13] found that increasing water stress by reducing soil moisture content from 70% to 40% of field capacity causes the percentage increase number of indigenous endomycorrhizal spores taken from salak roots to be higher. Allen et al. [14] and Wu and Zou [15] obtained number of mycorrhizal spores increased significantly with reduced rainfall or increased soil dryness. The higher presence of spores in the dry season is related to plant phenology [16]. When photosynthesis is reduced due to stomata closing, there is a decrease in carbon flow to the roots. Low carbon availability in roots stimulates the sporulation or formation of spores [17]. On the other hand, lower sporulation occurs in the rainy season, because in the rainy season the moisture content and humidity are high, cause anaerobic conditions that can inhibit the development of AMF due to AMF is an obligate aerobic microorganism [18]. Escudero and Mendoza [19] found *Tenuis lotus* is a mycotrophic legume that is tolerant to flooding, if it is submerged in flood for 7 months it causes a decrease in root colonization by mycorrhizae which indicates a decrease in the mutualistic symbiotic process.

The abundance and activity of mycorrhizae on plant roots is largely determined by environmental factors, especially drought [4, 20-23], and mycorrhizal species [24-26]. Fertile soil with sufficient water content reduces root colonization by mycorrhizae, whereas drought causes the level of root colonization and the effectiveness of P, N and K nutrient uptake by mycorrhizae increase [14, 21-22, 27-29]. Leal et al. [30] reported that the number of endomycorrhizal spores of the genus G*lomus* and *Acaulospora* in drought-affected *Eucalyptus camaldulensis* plants increased more than 300 times compared to plants grown on sufficient water content, but the increase in the number of spores of the endomycorrhizal genus *Scutelospra* was significantly lower, while Mathimaran et al. [31] found that moderate drought level caused root colonization by arbuscular mycorrhizal increase sharply with an increase in root range of up to 80%, but at severe drought level plant growth was inhibited and mycorrhizal colonization ability also decreased significantly.

This study aimed to determine the effect of water stress and mixed types of AMF genus from cocoa roots on spore propagation and root colonization used maize as a host. Corn was chosen as the host plnat because it is an excellent host for the development of mycorrhizal hyphae and spores [10]. In addition, maize has a short age, high adaptability, especially in dry land, and its root system develops rapidly so that it is very suitable for symbiosis with AMF.

2. Material and methods

2.1. Study site and material

Samples of AMF were taken from cocoa roots in 3 cocoa production centers in the Province of Bali, Indonesia, i.e., Mendoyo District Jembrana Regency, Selemadeg Timur District Tabanan Regency and Kubu Tamabahan District Buleleg Regency. Isolation and identification of AMF spores was carried out at laboratory, while spore propagation was conducted at Glass House, Farm Station, Faculty of Agriculture, Udayana University, Denpasar, Bali, from October 2021 to January 2022.

Materials used include corn seeds "Local Bali variety" as the host plant, aquades, 60% glucose, 10% KOH, 3% H_2O_2, 1% HCl, lactoglycerol, and trypan blue. Required tools include scissors, stereo microscope, compound microscope, centrifuge machine, centrifuge tube, ose needle, petri dish, autoclave, object glass, glass cover, and a set of sieve spores with hole diameter 1 mm, 500 µm, 212 µm, 106 µm and 53 µm.

2.2. Experimental design

The study used a 2-factors Randomized Block Design (RBD) and 3 replications. The first factor was water stress consisting of 3 levels, i.e., without water stress as a control, soil moisture content 100% of field capacity (W_0), light water stress, soil moisture content 70% of field capacity (W_1), and heavy water stress, soil moisture content 40% of field capacity (W_2). While the second factor was mixed type of AMF genus consisting of 3 levels, i.e., inoculant of genus *Glomus* only (G_g), mixed inoculants of the genus *Glomus* + *Acaulospora* (G_a), and mixed inoculants of the genus *Glomus* + *Scutelospora* (G_s). Thus, there were 9 combination treatments and 27 experimental pot units.

2.3. Determination of water stress level

Calculation of field capacity of the soil moisture content is done by watering the experimental pot containing media with excess water (watering volume is recorded) then left for one day (24 hours). Dripping water from excess watering is accommodated in container, then the soil moisture content of 100% field capacity is calculated by means of the volume of watering minus the volume of dripping water, while for the 70% and 40% soil moisture content is calculated by converting the volume of 100% of the known field capacity into 70% and 40% of field capacity.

2.4. Soil sampling and isolation of the genus AMF

The soil samples containing spores of AMF genus were taken from rhizosphere area around stem of cocoa tree (30 cm distance from the base of the stem and 0-30 cm depth from the soil surface). Spore isolation was done using wet screening technique followed by centrifugation technique according to Brunndret *et al*. [32]. The soil sample of 100 g was dissolved in 1,000-1,200 ml of water and stirred evenly, then filtered in a set of filtrations with diameter holes size from small to large i.e., 1 mm, 500 µm, 212 µm, 106 µm, and 53 µm, respectively. The dissolution and filtration were repeated 2-3 times, and then the rest of the soil was poured on the top filter. The top filter was then sprayed with tap water to facilitate the filtered material to pass. After filtering process on the top screen was completed, then proceed to the second filter, third, and so on. The remaining soil in 500 µm, 212 µm, 106 µm and 53 µm sieves were transferred to centrifuge tubes, then aquades were added 25-40 mL and centrifuged at 2,000 rpm for 5 min. The supernatant of the centrifuge process was thrown away and 60% glucose was added. The centrifuge tube was sealed and then centrifuged at 2,000 rpm for 1 minute. After 1 minute, the sugar-containing supernatant in each sieve was rinsed with water on sieve with diameter of 53 µm and the result placed in a petri dish then the spores are isolated. The isolated spores were identified and separated according to the genus *Glomus*, *Acaulospora* and *Scutelospora* and then placed in a carrier medium of zeolite granules which had been sterilized by heating at 121˚C in an autoclave for 30 minutes.

2.5. Growing host plants and AMF spore propagation

Corn host plants were planted in plastic pots with a volume of 2,5 kg using soil media. In a plastic pot which is planted the host plant, 50 spores were inoculated and mixed evenly in 500 g of granular zeolite carrier medium. For the level of inoculant treatment contain genus *Glomus* only (G_g), in 500 g of carrier medium was inoculated 50 spores of the genus *Glomus*, for the level of mixed inoculant treatment of genus *Glomus* + *Acaulospor*a (G_a), in 500 g of carrier medium was inoculated 25 spores of genus *Glomus* and 25 spores of genus *Acaulospora*, while for the mixed inoculant treatment level of the genus *Glomus* + *Scutelospora* (G_s), in 500 g of the carrier medium was inoculated 25 spores of genus *Glomus* and 25 spores of genus *Scutelospora*. The carrier media that has been inoculated with spores is then put into a plastic pot along with the soil planting medium.

The implementation of the AMF propagation was carried out according to the method of Rai et al. (2018), i.e., the order of placing soil media and spore-carrying media in plastic pots is that at the lowest layer is filled 1.25 kg of soil media and then on top of it placed 250 g of granular zeolite carrier media (in which AMF spores have been inoculated according to the level of treatment). The layer above it was placed again as much as 1.25 kg of soil media, after that on top of the soil in the center of the pot was placed 250 g of granular zeolite carrier media. After that, corn seeds were planted in the middle of the pot (3 seeds per pot) and arranged in such a way that they were in direct contact with AMF spores at the carrier media, then the seeds were covered with a thin layer of soil. At the age of 5 days after planting, thinning was carried out by selecting 1 young corn tree with the best growth. Watering until the age of 2 weeks after planting was carried out without water stress (all the pots were watered until the soil moisture content was 100% field capacity), then water stress treatment was given by adjusting watering according to the predetermined water stress treatment level. At the age of 6 weeks after planting, corn plants were cut (topping) half of the plant height, aiming to stimulate AMF to form spores. In topping, the host plant and AMF will get stress. After that, the host plant will die and AMF will try to defend itself where the hyphae will shrink and form spores. One week after topping, the roots of the host plant were dismantled, then observed the number of spores after propagation, percentage increase in the number of spores, and root colonization by AMF.

2.6. Observation number of spores, root colonization and growth of host plant

The number of spores reproduced was calculated by observing the isolates under a microscope, the percentage increase in the number of spores was calculated by the formula for the number of spores reproduced minus the number of initial spores (50 pieces per pot) divided by the number of initial spores, while the calculation of the percentage of root colonization was done using slide method according to Giovannetti and Mosse [33] by the formula: percentage of colonization root is the number of colonized roots divided by the total of all roots observed multiplied by 100%. Levels of root colonization are classified into 5 classes, i.e., very low (root colonization 0-5%), low (root colonization 6-25%), moderate (root colonization 26-50%), high (root colonization 51- 75%), and very high (root colonization 76-100%).

Observation of root colonization was done by root staining with trypan blue. Root staining was preceded by root washing until clean and then roots in one point were cut 2-5 cm length. After that, 20 pieces of 2-5 cm root length were put into test tube; added 10% KOH until all part of roots were submerged, heated at 250 °C for 10 minutes in the microwave and then stored for ± 12 hours at room temperature. After 12 hours KOH was removed by washing the roots with tap water (washing done 3 times) then added 3% H_2O_2 and stored for 12 hours at room temperature. After 12 hours H_2O_2 was removed by washing the roots with tap water (washing done 3 times), then added 1% HCL and stored for 12 hours at room temperature. After 12 hours HCL was thrown away, added trypan blue, heated at 250 °C for 5 minutes in the microwave and then stored for 12 hours at room temperature. Then, trypan blue was removed, added lactoglycerol and heated at 250 °C for 5 minutes on the microwave for 12 hours at room temperature. Finally, the roots were taken with tweezers, placed in a row and observed under microscope to calculate the presence or absence of root colonization by AMF.

In addition, observations were made on the growth of the host plant including the number of leaves, plant height, stem diameter, root length, and total oven dry weight of the host. The number of leaves, plant height and stem diameter at 5 cm above the surface of the media were measured shortly before the host plant was topped, root length was measured from the base of the stem to the tip of the longest root, while the total oven dry weight was the sum of the shoot and root oven dry weight of host.

2.7. Statistical analysis

The collected data was analyzed using Analysis of Variance (Anova). If there was a significantly difference between treatments then tested further with *Least Significance Different* (LSD) Test.

3. Results and discussion

3.1. Number of spores reproduced and root colonization

The results of the analysis of variance showed that the interaction between water stress and mixed types of the AMF genus had no significant effect on all observed variables. Water stress treatment and mixed type of AMF genus in a single factor significantly affected the number of spores reproduced and the percentage increase in number of spores after propagation, but had no significant effect on the percentage of root colonization by AMF.

In the treatment of water stress, the higher the water stress or the lower the soil moisture content from field capacity, the higher the number of spores produced and the percentage increase in the number of spores. In Table 1 it can be seen that the highest number of spores after propagation was obtained at heavy water stress (W_2), which was 6,713.40 pieces/pot and was significantly higher than W_1 and W_0. The increase in the number of spores after propagation compared to the number of spores inoculated (50 pieces/pot) in W_2 and W_1 reached 13,326.82% and 11,633.59%, respectively. These data indicated that water stress greatly affects the ability of indigenous AMF to reproduce. Increasing water stress by reducing soil moisture content from 100% of field capacity to 70% and 40% of field capacity gave a greater increase in the number of spores, even the percentage increase in the number of spores at a soil moisture content of 40% of field capacity compared to soil moisture content of 100% of field capacity up to 1,914.39%. The results of this study were in accordance with the result of Rai et al [11] that in propagating of indigenous endomycorrhizal taken from rooting area of snake fruit, the highest percentage increase of the spore after propagation was at soil moisture content of 40% of field capacity. Sadhana [4] and Wu and Zou [15] stated that the abundance and activity of mycorrhizae in the soil rhizosphere layer is largely determined by the level of drought. According to Quiroga et al. [34], drought increased the number of spores on maize roots and drought-tolerant cultivars were colonized higher than drought-sensitive cultivars. Similarly, Leal et al. [30] reported that the number of endomycorrhizal spores of the genera *Glomus* and *Acaulospora* in *Eucalyptus camaldulensis* plants exposed to drought increased more than 300 times compared to controls. Based on the results of this study, to obtain a large number of spores as an ingredient for making biofertilizer, the best way to propagate spores is by giving heavy water stress treatment with a soil moisture content of 40% of field capacity.

Table 1 showed the percentage of root colonization at the level of heavy water stress (W_2) and light water stress (W_1) was not significantly different from the percentage of root colonization in the control (W_0) with a value of 100%, respectively. This showed that all samples of host plant roots in this study were highly colonized by AMF (Nurhandayani et al., 2013). According to Chareesri et al. [23] and Kumar and Tapwal [35], the degree of root colonization by mycorrhizae largely determines the level of symbiosis between mycorrhizae and their hosts. The higher the level of colonization, the higher the symbiosis that occurs. In this study, high root colonization by AMF did not only occur in propagation using maize as its host, but also occurred naturally in cocoa roots, as evidenced by the level of root

colonization at three sampling locations for isolation and identification of AMF genus which also reaches 100%. The very high percentage of root colonization on cacao and corn host plants which reached 100% indicated that AMF derived from cocoa roots had a high adaptability so that it was suitable as a source of inoculants for the manufacture of biofertilizers.

Table 1 The number of spores reproduced, percentage increase number of spores and root colonization after propagation due to influence by water stress and mixed type of AMF genus

Treatment	Number of spores after propagation (pieces)	Percentage increase number of spore after propagation (%)	Percentage of root colonization (%)
Water stress (W)			
W_0	5,060.10 c	10,020.24 c	100
W_1	5,866.80 b	11,633.59 b	100
W_2	6,713.40 a	13.326.82 a	100
LSD 5%	568.80	1.037.60	ns
Mixed type of genus AMF (G)			
G_g	5,306.70 b	10,513.38 b	100
G_a	6.069.90 a	12.039.84 a	100
G_s	6,263.40 a	12.427.77 a	100
LSD 5%	568.80	1,037.60	ns

Noted: In the same column, the numbers followed by the same letter for the treatment of water stress and mixed type of genus AMF showed no significant effect based on LSD at 95 % confident level; ns = not significantly different.

In the mixed type of the AMF genus treatments, the highest number of spores after propagation was obtained in the mixed inoculant of the genus *Glomus* + *Scutelospora* (G_S) with a value of 6,263.40 pieces, but it was not significantly different from the mixed inoculant of the genus *Glomus* + *Acalospor*a (G_a), and the lowest was in single genus *Glomus*(G_g) inoculant with 5,306.70 pieces. Thus, the percentage increase in the number of spores after propagation compared to the number of spores inoculated (50 pieces per pot) on G_s and G_a reached 12,427.77% and 12,039.84%, respectively. The results of this study showed that in multiplying AMF spore, spores inoculation with a mixture or consortium between genera gave a significantly higher increase in the number of spore than single genus inoculation. This result was in line with Pellegrino et al. [36] that the consortium of AMF causes higher symbiotic activity, thereby increasing the yield of *Medicago sativa* plants. However, the percentage of root colonization of host plants by mixtures between genera was not significantly different with a single genus, which was both very high reaching 100%, which indicated that the symbiotic activity of AMF with plants was as intensive whether the spores were inoculated as a mixture of ganus or only one genus.

3.2. Host plant growth

The results of the analysis of variance showed that the interaction between water stress and mixed types of AMF genus had no significant effect on all observed growth variables of the host plant. Water stress treatment had a significant effect on plant height and stem diameter, while the mixed type of AMF genus treatment had no significant effect on all host plant growth variables.

In water stress treatment, the higher the water stress, the lower the height and stem diameter of the host plant. Table 2 showed that the highest host height and stem diameter were obtained at the treatment level without water stress/control (W_0), which were 132.22 cm and 0.78 cm, respectively, while the lowest were obtained at heavy water stress with values 122.67 cm and 0.70 cm, respectively. Although the plant height and stem diameter were highest at W_0, the total oven dry weight of the host plant at W_0 (6.82 g) was not significantly different from W_1 (7.25 g) and W_2 (7.81 g). The total oven-dry weight of the host plants was not significantly different between W_0 with W_1 and W_2 related to the number of leaves and root length that were not significantly different among those levels of treatment (Table 2). The number of leaves did not significantly different cause the photosynthesis process at the level of light and heavy water stress to be not lower than those of without water stress/control, while root lengths did not significantly different cause nutrient and water absorption at the level of light and heavy stress to be undisturbed, so that the photosynthate

produced in the control was not significantly different from light and heavy water stress. In addition, the total oven dry weight of the host plants at W_1 and W_2 was not significantly different from the control and was also associated with very high root colonization by AMF. The very high root colonization which reached 100% in W_1 and W_2 (Table 1) indicates a very high AMF and host plant symbiosis so that the water and nutrient requirements of the host plant can be fulfilled well even under water stress conditions.This is in accordance with the research results of Manurung et al. [37] that soil moisture content of 80% and 60% of field capacity in endomycorrhizal inoculated rubber nurseries caused the total dry weight of seedlings to be significantly higher than at soil moisture content of 100% of field capacity. The same thing was stated by Mathimaran et al. [31] that, water stress causes root colonization by arbuscular mycorrhizae increases sharply with an increase in root range of up to 80%. The high increase in the number of spores under heavy water stress with a soil moisture level of 40% of field capacity compared to control indicated that the adaptability of AMF originating from cocoa roots to drought was very high. This high adaptability in this study was offset by its ability to prevent a decrease in the growth of indicator plants (maize), which was indicated by the total oven dry weight of the host plant which was not significantly different between W_0 with W_1 and W_2.

Table 2 Effect of water stress and mixed type of AMF genus on host plant growth

Treatment	Number of leaves per plant (pieces)	Plant height (cm)	Stem Diamater (cm)	Root lenght (cm)	Total dry oven of host plant (g)
Water stress					
W_0	5.61 a	132.22 a	0.78 a	61.22 a	6.82 a
W_1	5.94 a	124.72 ab	0.72 b	63.22 a	7.25 a
W_2	5.67 a	122.67 b	0.70 b	63.56 a	7.81 a
LSD 5%	ns	7.72	0.03	ns	ns
Mixed type of genus AMF					
G_g	6.00 a	124.17 a	0.72 a	63.39 a	7.04 a
G_a	5.56 a	125.00 a	0.71 a	62.11 a	7.75 a
G_s	5.67 a	130.44 a	0.69 a	62.50 a	7.09 a
LSD 5%	ns	ns	0.03	ns	ns

Noted: In the same column, the numbers followed by the same letter for the treatment of water stress and mixed type of genus AMF showed no significant effect based on LSD at 95 % confident level; ns = not significantly different.

The mixed type of the AMF genus treatment had no significant effect on the growth of the host plant, which was indicated by the number of leaves, plant height, stem diameter, root length and total oven dry weight of the host plant, which were not significantly different between single anoculants of the genus *Glomus* (G_g) and mixed inoculants *Glomus* + *Acaulospora* (G_a) and mixed inoculants *Glomus + Scutelospora* (G_S). The growth of host plants that were not significantly different among G_g, G_a and G_S was related to the very high percentage of root colonization by AMF which was 100% in G_g, G_a and G_s, respectively. Very high colonization of host plant roots indicated that AMF isolated from cocoa roots could have very good symbiosis with maize roots, and mycorrhizal roots expanded the absorption range resulting in high nutrient and water uptake and overall plant growth was not significantly different among G_g, G_a and G_S. Spore density and root colonization of host plants were largely determined by the suitability of mycorrhizae with host plants, environmental factors, and interactions between mycorrhizae and chemical compounds produced by host plants [38, 39]. Thus, root colonization that was not significantly was thought to cause plant growth on G_g to be not significantly different than that of on G_a and G_s because the ability of the host plant to absorb nutrients and water was equally well assisted by AMF. Similar results were presented by Chaeseri et al. [23] that increased colonization of arbuscular mycorrhizal fungi reduced the yield loss of rice (*Oryza sativa* L.) under drought conditions. The results of this study also showed that a mixture of genera (G_a and G_s) resulted in an increase in the number of spores which was significantly more than a single genus (G_g), but the mixture of genus did not increase the growth of the host plant. This means that in order to obtain a higher number of spores for inoculants in the manufacture of biofertilizer, it is better to use a mixture of genera due to provide the same good growth of host plants as a single genus.

4. Conclusion

There was no significant difference between the interaction of water stress and mixed species of the AMF genus on the number of spores reproduced and root colonization. The best way of propagation of AMF spores is by heavy water stress treatment with a soil moisture content of 40% of field capacity producing 6,713.40 spores or an increase of 13,326.82%. Mixed inoculants of the genus *Glomus* + *Scutelospora* gave the highest number of spores after propagation (6,263.40 pieces) or an increase of 12,427.77%.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared that there is no conflict of interest.

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