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Proline and Abscisic Acid Content in Droughted Corn Plant Inoculated with *Azospirillum* sp. and Arbuscular Mycorrhizae Fungi

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Key words: *Azospirillum* sp., Arbuscular Mycorrhizae fungi, Corn, drought, proline, abscisic acid (ABA)

INTRODUCTION

Under field conditions, plant generally undergoes water deficit due to water limitation in the plant roots area which resulted in lower water absorption. Transpiration rate that precedes water absorption by root will subsequently decrease the plant water content (Kramer 1983). Consequently, it will reduce plant turgor pressure and water potential. These conditions might disturb biochemical and physiological processes, hence resulted in anatomical or morphological changes of the plant.

Plants that undergo drought stress perform a physiological response such as accumulation of proline in the leaves. Proline accumulation usually more pronounce than other amino acids in the under drought condition plant. During the beginning of drought stress, proline content increase slowly, however it increase dramatically after the severe drought (Girousse *et al.* 1996; Yang & Kao 1999). Yoshiba *et al.* (1997) reported that the accumulation of proline was higher in the tolerant than in the sensitive plant. This implied that proline was able to support plant to recover after water stress and during rewatering (Peng *et al.* 1996).

Clawson *et al.* (1989) reported that under drought stress the plant usually enhance abscisic acid content (ABA) content in their leaves as well. ABA synthesis was started immediately after the plant was exposed to the dry media. This process reduces stomatal pores and finally the pores were close. After rewatering, the ABA concentration in the guard cell of the stomata reduces. This process subsequently increases the concentration of K^+ ion and turgor pressure results in the opening of stomata; hence, it increase photosynthesis process due to improvement of CO_2 supply. In many cases, plants that undergoes water deficit damage its cortex tissues and root. However, this will not be the case if the plant has a symbiosis relation with arbuscular mycorrhiza fungus (AMF). This is due to soil volume surrounding the plant can be explored by the root with AMF was approximately 12-15 cm^3 of soil (6-15 folded), while 1-2 cm^3 without AMF (Sieverding 1991). This means, symbiosis between plant and AMF will perform adaptable to water deficit.

The root of the plant with mycorryza can grow normally soon after drought period. This is due to AMF hypha is still able extract water in the microphores of water table in the soil, while the plant root can't. A wide spread of AMF hypha surrounding the root can help the plant to absorb more water (Osonubi *et al.* 1991). Another positive effect of AMF on the plant is its ability to improve phosphorus availability for the host plant (Sieverding 1991).

Another microorganism that has a role in plant growth promotion is *Azospirillum* that colonized in the intracellular of cortex and endodermis cells of the roots and *Azospirillum* can survive under the drought conditions (Michiels *et al.* 1989). *Azospirillum* sp. is able to improve absorption of N, P, K, and micronutrient, plant water status, plant dry weight, and yield of corn as well (Cosico *et al.* 1991).

Recently, there is lack of data for the role of AMF and *Azospirillum* to support physiological processes during the drought stress. This encouraged our group to investigate the

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role of AMF and *Azospirillum* in relation to proline and ABA accumulation in corn during the drought stress especially between the stage of flowering and seed filling.

MATERIALS AND METHODS

Inceptisol soil used for these experiments was characterized with silty loam texture and low fertility status. This soil was collected compositely from Cimanggu, Bogor, West Java, at 0-25 cm below the soil surface. No sterilization was carried out to this soil. The physical properties of the soil were: moisture content at field capacity with pF = 2.54 was 36.76% and at permanent wiling point with pF = 4.20 was 4.13%. Other physical properties of the soil were available water content, dry air water content, and soil dry weight at room condition were 32.63%, 11.71%, and 10,000 g, respectively.

To determine the stress conditions with 30% of available water content, we used the formula as follow:

Water content = (30% x available water content) + soil water content at permanent wiling point

This formula was important to determine soil weight for every polybag that will be used for drought treatments.

Wet weight of soil for every polybag was calculated by:

$$\text{Water content} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Dry weight}}$$

Based on the initial biological study using most probable number method (MNP), we found that the population of *Azospirillum* sp. was 3.30 x 10^6 cells per 100 g of soil, while infective propagule of AMF (spores, roots colonized by AMF and AMF hypha) was 6.069 propagules per 100 g of soil.

In this experiment we used Bayu variety corn seeds having 97% germination rate. The AMF inoculum that was used in the experiment was *Glomus manihotis* in the form of infective propagules. Liquid inoculums of *Azospirillum* sp. (Isolate number of Az.7) was given in the density of 10^8 of cell/ml. The plant materials, AMF inoculums and isolate of *Azospirillum* sp. were acquired from Center of Crop Biotechnology and Genetic Resources (BB Biogen), Bogor.

The experiments were carried out in glasshouse using Blok Randomize Design with two factors, i.e. (i) dosage of *Azospirillum* sp. notified by "A" with four levels of treatment (0, 0.5, 1.0, and 1.5 ml of *Azospirillum* sp. with concentration of 10^8 cells/ml for every polybag; and (ii) the dosage of AMF notified by "M" which also contained four levels of treatment (0, 12.5, 25.0, and 37.5 g of AMF per polybag). All treatments comprised of 16 combinations with 2 replications for every treatment. To obtain some correction factors of plant fresh weight, 16 polybags without plant were also added in the experiment.

Method. Ten kg of dry-air soil was sieved with 2 mm of soil siever and was loaded to the polybag. To facilitate watering, on every polybag, a pair of plastic tubes (0.5 inch of diameter) was installed in two different deep levels, i.e. 10 and 15 cm at different side of the polybag. We expected that water would spread evenly by using those two levels of tubes.

To support plant growth, the plant was fertilized using basic fertilizers one day prior planting. The basic fertilizers for every polybag were 0.7, 0.5, and 1.0 g of Urea, SP-36, and KCl, respectively. These three fertilizers were mixed with the soil prior to media loading in the polybag and were arranged in the glasshouse.

Before planting, corn seeds were sterilized using 0.1% of $HgCl_2$ (10 minutes) and washed using sterile water (5 times). The inoculation of *Azospirillum* sp. was carried out by spraying the inoculums to the soil around the seedbed with the dosage that has been explained before, while for AMF, the inoculums were given as infective propagule by spreading them under the seed during seed planting. Three seeds were planting for every polybag in 5 cm depth.

After a week, two homogenous seedlings were chosen out of three seedlings. Within 44 days after planting (before flowering), the plants were grown under normal conditions with water content was maintained nearly constant to about 100% of field capacity (FC). Subsequently in 45-55 days at flowering and seed filling stage, drought stress was given by watering 30% of water availability to all plants. Water content of media was controlled by gravimetric method to determine additional water. The increased plant weight for correction factor was calculated between 14 up to 49 days plant. As comparison to this method, the "Bouyoucos moisture meter" was also used. After 55 days, i.e. after seed filling stage, the plants were harvested.

In this experiment, proline and ABA content of the plant were measured at the fully expanded leaves of the 55 days plant by using the 4th leaf from the tip of the plant. Proline was analyzed based on Bates *et al*. (1973) method by using pure proline as the standard. Acid ninhydrine was prepared by preheating 1.2% of ninhydrine into a mix of 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid. The mixture was then stored at 4 ºC, which was stable within 24 hours. Proline of approximately 0.5 g of fresh leaves was extracted with 10 ml 3% sulfosalicilic acid, then was filtrated using 2 sheets of Whatman paper no 42. About 2 ml of filtrate was reacted with 2 ml of acid ninhydrine and 2 ml of glacial acetic acid in test tube for 1 hour at 100 ºC and the reaction was abolished in icebath. The mixture was extracted using 4 ml toluene and was shake using test tube stirrer for 15-20 second. Chromophore in the solution was warmed at room temperature and the absorbance was measured with spectrophotometer at ë = 520 nm. For this measurement, toluene was used for the blank sample. Proline content (ì mol/g) was determined by using standard curve and calculated based on the fresh weight sample (Bates *et al*. 1973) as follow:

$$\frac{\text{ìmol prolin/g}}{\text{fresh weight}} = \frac{[(\text{ig proline/ml x ml toluene})/115.5\ \text{ig/ìmol}]}{(\text{g sample})/5}$$

ABA content was measured using Elisa Kits method and determined by using HPLC model 510.

AMF Colonization in the Root. AMF colonization in the root was analyzed using fuchsin acid staining method and colonized roots were calculated using slide length method (Gerdemann 1975): (the number of infected roots/total number of observed root) x 100%

Nitrogen fixation was determined from the fresh root sample by using acetylene reduction activity (ARA) method and was analyzed with gas chromatography. ARA quantification was as follow:

$$\text{ARA (i mol g}^{-1}\text{jam}^{-1}) = \text{Ethylene molecule weight (EMW) x time of incubation (t) x fresh root weight (FRW) x Standard} \quad X$$

Data Analysis. The effects of each treatment and their interaction on response variables were analyzed by using univariate analysis. Advance analysis was carried out to understand specific response of the treatments using DMRT test at 5% level.

RESULTS

Proline. The interaction of *Azospirillum* and AMF was significantly influenced proline content of corn plant subjected to drought stress (Table 1). Single effect of *Azospirillum* inoculation was able to improve proline content of leaf although under lower dosage treatment (0.50 ml/polybag) as compared to control (without inoculation) plant. The same response occurred at the AMF treatment with dosage of 12.50 g/polybag. On the other hand, if higher dosage of *Azospirillum* was applied, no significantly different showed in the proline content (P = 0.05). In addition, the application of AMF with higher dosage caused the decrease of proline content.

The different combination of *Azospirillum* and AMF gave different effect on proline content and the different dosage of *Azospirillum* and AMF showed inconsistent effect on proline content. The effects tended to be antagonist between *Azospirillum* and AMF. This can be seen from the data about the interaction effect of *Azospirillum* (0.50 ml/polybag) with AMF (12.50 and 25.00 g/polybag) which was not significantly different (P = 0.05) from the plant without inoculation. However, if the AMF dosage was improved (37.50 g/polybag) the proline content even decreased. In the same way, if a lower dosage of AMF combined with medium (1.00 ml/polybag) and high dosage (1.50 ml/polybag) of *Azospirillum* was also not significantly different (P = 0.05) from control, and if the dosage was improved further it also caused the decrease of proline content.

Abscisic Acid (ABA). The ANOVA data indicated that inoculation of *Azospirillum* and AMF significantly (P = 0.05) influenced ABA content of corn leaf that was subjected to drought stress during flowering and seed filling (Table 1). ABA is a hormone that has a special role as chemical signal to the plant organs that undergoes physiological drought stresses. Without inoculation of either *Azospirillum* or AMF, the plant subjected to drought stress had maximum ABA content 455 ñmol/g of fresh weight as compared to other treatments. With a single treatment, the inoculation using various dosage of *Azospirillum* decreased ABA content more than that of using AMF with low and medium dosage (12.50 and 25.00 g/polybag AMF respectively). The combination of *Azospirillum* and AMF also decreased of ABA content as compared to control plant. Meanwhile, the increase of *Azospirillum* or AMF dosage did not affect the ABA content.

DISCUSSION

The inoculation of *Azospirillum* sp. with a particular dosage was able to improve proline content of corn subjected to drought stress during the flowering and seed filling. This phenomenon may be associated with the role of *Azospirillum* which is able to fix nitrogen compound from the air (Table 1), and consequently influenced the accumulation of proline content. This process might be able to support the plant to be more adaptable to severe drought stress when water availability was only about 30%. The increase of proline content was might associated with the development of AMF hypha which assisted the plant to extract water as well as nutrients from the dry soil. This data was in accordance to that of Ruiz-Lozano *et al.* (1995). They found that proline content was

Table 1. Response of *Azospirillum* dan FMA *G. Manihotis* innoculation on root colonization by FMA, nirogen uptake, proline and ABA content of maize under drought conditions during flowering and pod filling

Azospirillum (ml/polybag)	FMA (g/polybag)	Root colonization (%)	Fixation N (çmol/g fresh root/h)	Proline content (çmol/g fresh weight)	ABA content (ñmol/g fresh weight)
0	0	11a	7a	95a	455c
	12.50	64b	15b	115b	265b
	25.00	62b	14b	90a	250b
	37.50	64b	13b	105ab	155a
0.50	0	13a	12b	120b	125a
	12.50	63b	17bc	115b	125a
	25.00	74b	16bc	115b	120a
	37.50	65b	14b	95a	100a
1.00	0	25a	19c	115ab	90a
	12.50	44ab	16bc	130b	85a
	25.00	46b	16bc	105a	85a
	37.50	76c	19c	105a	75a
1.50	0	18a	16bc	125b	75a
	12.50	29a	19c	110ab	75a
	25.00	47b	19c	100a	65a
	37.50	76c	21d	125b	60a

higher (119.60 nmol/g fresh weight) in drougted salad that had been inoculated by *Glomus deserticola*, while it was only 16.20 nmol/g in the drougted salad without inoculation.

According to Fidelibus *et al.* (2001) the effect of AMF on adaptability of host plant to drought stress is probably a secondary effect due to the increase of nutrient status of the host plants. Subramanian and Charest (1999) reported that AMF colonization on corn plant was able to stimulate the activation of principle enzymes that involve in nitrogen assimilation such as *nitrate reductase* and *glutamate synthetase* especially during drought conditions. The improvement of this enzyme activity can change and increase nitrogen content of the plant which resulted in increase of proline content. Consequently, this situation can improve plant adaptability to drought stress and plant recovery soon after rewatering.

On the contrary, the plants without inoculation of *Azospirillum* and AMF showed severe stress due to drought (Figure 1) indicated by wilting and rolling leaves. These plants also had a higher ABA content in their leaves. The increase of ABA content in the plant in response to drought stress has been reported many authors such as Alves and Setter (2000).

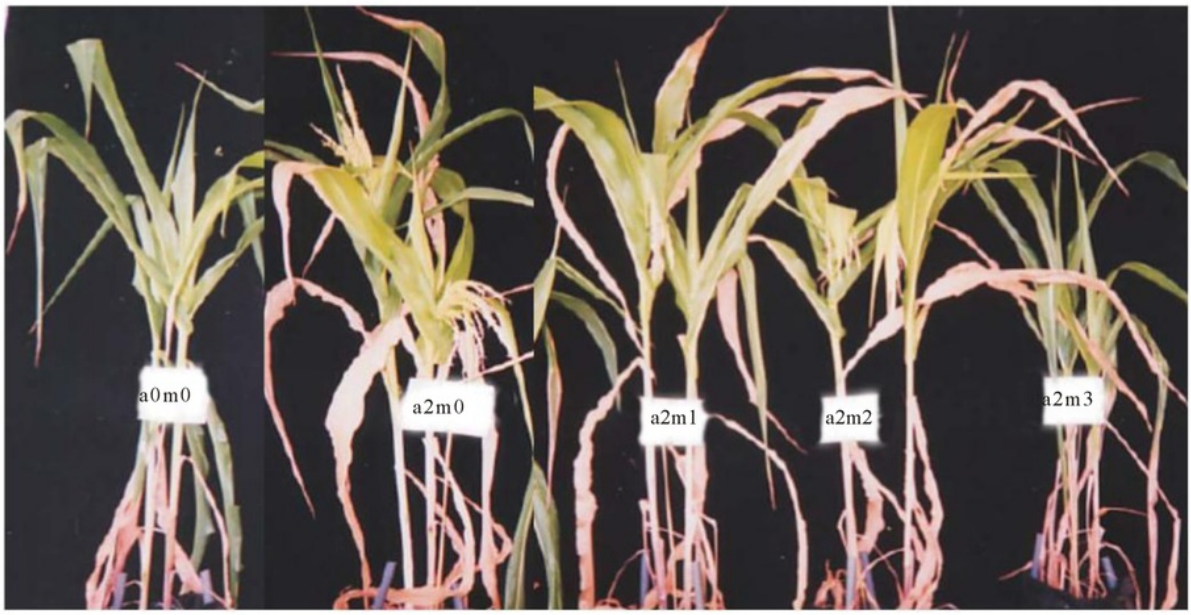

Figure 1. Maize plants that were grown under drought stress in the glasshouse using polybag with different treatments of *Azospirillum* sp. (a0: control, a2: 1 ml of 10^8 cell/ml) and arbuscular mycorrhizae (m0: control, ml : 12 g of mycorrhizae, m2: 25 g of mycorrhizae, m3: 37.5 g of mycorrhizae).

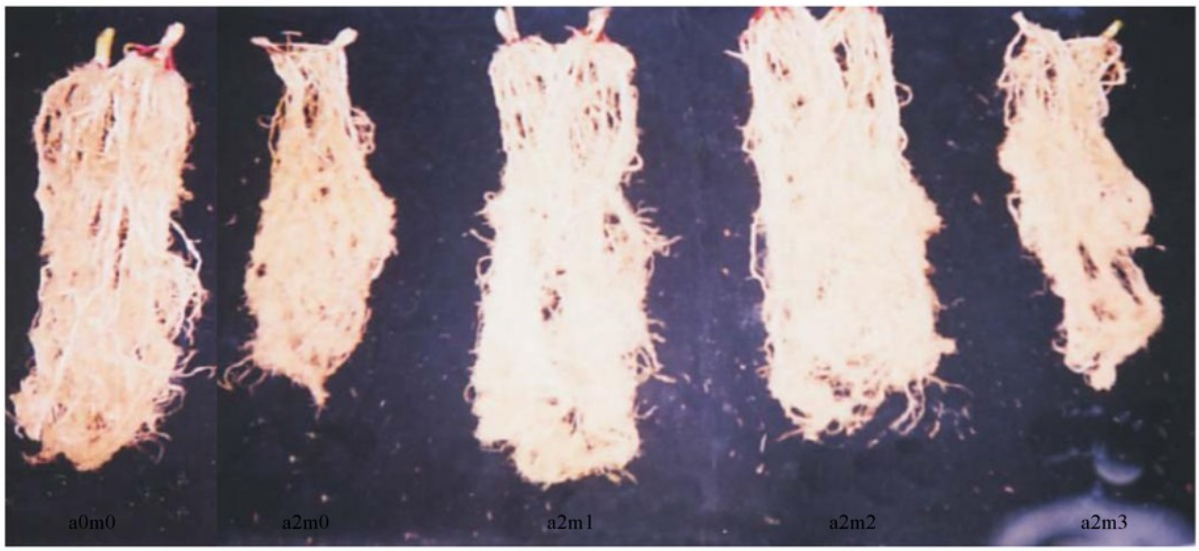

Figure 2. The root of maize that were grown under drought stress with different treatments of *Azospirillum* sp. (a0: control a2: 1 ml of 10^8 cell/ml) and arbuscular mycorrhizae (m0: control, ml : 12 g of mycorrhizae, m2: 25 g of mycorrhizae, m3: 37.5 g of mycorrhizae).

According to Mansfield and McAinsh (1995), the plant under drought stress generally increase its ABA content more than 20 times e.g. up to 8 femtogram per cell (80^{-15} g/cell). During the drought stress, roots synthesize ABA and it transports through plant xylem to the leaves which subsequently resulted in stomatal closure. ABA induces stomatal closure through an inhibition of proton pump activity that depend on ATP abundance in plasma membrane of guard cells. ABA works on the surface of intercellular of cell membrane prevent the inclusion of K^+ to the guard cell. Hence, K^+ and consequently water exclude from the guard cells which cause the reduction of turgor pressure and finally stomatal closure. Ordinarily, proton pump excludes the proton from the guard cells where at the same time the K^+ is accumulated to the guard cells. This process reduced the osmotic pressure in the guard cells which induces absorption of water and finally stomatal opening. Another experiment has also indicated that plasma membrane reduced turgor pressure by accelerated Ca^{2+} transporting into the cell. Ca^{2+} and phosphoinositol have a role to activate genes that are required to synthesize ABA (Salisbury & Ross 1995).

The inoculation of *Azospirillum* sp. with a certain dosage to corn plant subjected to drought stress during flowering and seed filling was able to reduce ABA content in the plants. This probably was associated with the function of *Azospirillum* sp. in nitrogen fixation (Table 1) which influenced nitrogen content in the soil and plant. Orcutt and Nilsen (2000) reported that ABA concentration inside the plants might be influence by the level of nitrogen source (NO_3^- or NH_4^+). In addition, various contents of Zn, K, and P inside the plant were also influenced ABA concentration in the plants.

The reduction of ABA content in droughted plant inoculated by AMF may be in associated to the development of AMF hypha which assists plant to extract water and essential nutrients under dry conditions. Similar result has also been reported by Duan *et al.* (1996), Ebel *et al.* (1997), and Goicoechea *et al.* (1997) who found that application of AMF was able to reduce ABA content of droughted plant. This results suggested that inoculation of AMF to the drougted plant is able to alleviate the strained by manipulation of stomatal conductance so that the stomata are still remained open for the longer period.

This experiment indicated as well that the inoculums of *Azospirillum* sp. and AMF can work synergically and was able to improve proline content and reduce ABA concentration in the corn plant subjected to drought stress during flowering and seed filling. Trotel-Aziz *et al.* (2003) reported that there is good correlation of proline accumulation and ABA concentration changes. The phytohormone ABA may work at the beginning site of enzyme activity of Ä[1]*-pyrroline-5-carboxylate synthetase* (P5CS), as the response to induce substrate during proline synthesis or at the end of enzymes activity of P5CS which associated to the level of *proline dehydrogenase* (PDH).

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