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Genetic diversity and morphological responses of *Capsicum annuum* varieties under aluminum stress

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Abstract. *Andriyani, Jadid N. 2021. Genetic diversity and morphological responses of* Capsicum annuum *varieties under aluminum stress. Biodiversitas 22: 2576-2582. Capsicum annuum* is one of the most cultivated vegetable crops in the world. Its fruit contains various beneficial metabolites which possess antioxidant activities. Fertilizers play important role to increase plant productivity. However, excessive use of fertilizers could harm the environment. Heavy metals, including aluminum (Al), contamination have been reported to negatively affect plant production and inhibit root growth. *C. annuum* var Ciko, Lingga, Lembang1, and Tanjung2 are commonly cultivated in Indonesia. However, the genetic diversity of those plants and their responses to Al stress condition are still unknown. Therefore, our study aimed to analyze the genetic diversity of four *C. annuum* cultivars and to observe their morphological responses under Al stress conditions. The genetic diversity was analyzed using five universal inter-simple sequence repeat (ISSR) primers. Our results showed that the PIC values ranged from 0 (primer 825) to 0.6375 (Primer 9). The UPGMA phylogenetic construction also demonstrated that the cv. Lembang1 is closely related to the cv Lingga. Meanwhile, cv Tanjung 2 is closely related to the cv. Ciko. Each cultivar also performed different morphological responses under Al stress conditions. Our data showed that Al affected significantly root growth but not affected their plant height. Aluminum at 100 ppm significantly decreased root length of cv. Lingga.

Keywords: Aluminium, *C. annuum*, genetic diversity, ISSR, morphological responses.

INTRODUCTION

Capsicum (*Capsicum annuum*) is a horticultural crop that possesses high economic value (Rukmana 1995). It is consumed worldwide and used in some industrial domains, including food and health industries (Khan and Leskovar 2006). This plant is widely used in the food industry because of its alkaloid content, namely capsaicin, which is responsible for its spicy taste (Hoffman et al. 1983). In addition, Capsicum is also a source of vitamin C and vitamin B (Ganguly 2017). Moreover, this crop contains valuable essential oil, capsitol. Capsitol is used to substitute the eucalyptus oil to reduce stiff shortness of breath, itching, and rheumatism. The flavonoid content of this crop is also useful to inhibit inflammation (Rukmana 2002). Due to the high utilization of capsicum plants, generation of new varieties with elite phenotypes becomes important. Some of the varieties that are currently developed in Indonesia include var. *Ciko, Tanjung 2, Lingga and Lembang 1*. These four varieties have different productivity. For instance, var. Ciko has a productivity of 13.4-20.5 t ha^{-1}, and that of Lembang1 is 15.6-19 t ha^{-1}, Lingga is 13.4-20.5 t ha^{-1}, and Tanjung2 is 12 t ha^{-1} (Balitsa 2018). Although there are many varieties of *Capsicum* plants that are being cultivated in Indonesia, their genetic information is still limited. Therefore, conducting genetic diversity analysis of important crops is necessary for the development of new variety and conserving the genetic resources (Sun et al. 2008). The study of genetic diversity can be done using many molecular markers.

Molecular markers have been widely used to study the genetic variations among genotypes (Kaur et al. 2015), population studies (Ng et al. 2015), phylogeny (Acharya et al. 2004), and genetic mapping (Yagi et al. 2017). Currently, many molecular markers have been developed including Amplified polymorphic length (AFLP), Restriction fragment length polymorphism (RFLP), and Simple sequence repeat (SSR), Random amplified polymorphic DNA (RAPD), and inter simple sequence repeat (ISSR) (Nadeem et al. 2017). ISSR is a highly polymorphic marker that has been used for genetic diversity studies (Xing et al. 2015). This marker is cost-effective, high productivity, and is not affected by environmental factors (Das et al. 2017; Ray et al. 2019).

Genetic diversity is one reason for the different responses of plants to environmental conditions (Rukmana 2005). Environmental conditions are a determining factor for the productivity level of chili plants. Indonesia is a tropical country where most of the land is acidic, 148 million hectares are considered as dryland, and 102.8 million hectares are acidic dryland (Mulyani et al. 2004). Acidic soil is defined as soil where its top layer possesses pH of less than 5.5 (He et al. 2019). Unpredictable climate change and relatively high rainfall in most Indonesian regions have resulted in an intensive level of alkaline soil leaching. It leads to an increase in soil acidity (Subagyo et al. 1998).

Aluminum (Al) is a heavy metal that can disturb plants in the form of trivalent cations (Al^{3+}). Al quickly binds to the apoplast and it is also deposited in the plant vacuole. High Al accumulation will trigger the production of free

radicals in plants. The reactive oxygen species (ROS) will initiate the lipid peroxidation of the cell membranes (Panda and Baluska 2015). Al also interferes with the absorption of nutrients from soils (Moustaka et al. 2016). In acidic soil, free aluminum forms insoluble aluminum phosphate which causes a phosphate deficiency (Handayanto 2017). Al^{3+} will bind to the $H_2PO_4^-$anions and precipitate them as Al-phosphate (Raharjo et al. 2007).

Several studies have shown that Al stress influences plant physiology, morphology and molecular aspects. Karimaei and Poozesh (2016) revealed that Al stress reduced the chlorophyll content in *Spinacia oleracea*, affecting the photosynthesis process. Heavy metals can induce damage that can inhibit the photosynthesis process (Jadid et al. 2017). The photosynthetic process can be inhibited because heavy metals replace Mg^{2+} in chlorophyll molecules (Kuepper et al. 1998). Heavy metals can also replace enzyme cofactors, thereby reducing the activity of photosynthetic enzymes (Shanmugam et al. 2012). High accumulation of aluminum stress in *Stenocalyx dysentericus* was reported to cause root growth inhibition (Rodrigues et al. 2016). Another study has demonstrated an increase in proline synthesis during Al stress (Mantovanini et al. 2019). In addition, like in other abiotic stress conditions, plants regulate their responses through a dynamic gene expression (Jadid et al. 2018). Based on the above background, this study evaluated the genetic diversity of four varieties of capsicum using ISSR markers and the morphological responses of four varieties of capsicum during Al stress conditions.

MATERIALS AND METHODS

Study area

Capsicum cultivation and Al stress condition were conducted at the greenhouse facility of the Urban Farming area of the Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia. Meanwhile, the morphological responses of capsicum against Al stress were evaluated at the Laboratory of Bioscience and Plant Technology, Department of Biology, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia.

Procedures

Plant materials, cultivation and Al stress condition

Four capsicum varieties were used in this study. It includes capsicum var. *ciko, lembang1, lingga and tanjung2*. The seeds were soaked using tap water overnight. Subsequently, the seeds were germinated in the polybags containing soil and compost media with a ratio of 1: 1 according to the method described previously (Jadid et al. 2017; Setiadi 2006).

The soil media for Al stress treatment was dried for 24 hours and then put in a 15 cm x 15 cm polybag (Dogic et al. 2017). Al stress treatment was carried out after the plants aged 2 weeks. Before mixing Al into the soil, a stock of 1 L $AlCl_3$ 1000 ppm was made. The Al concentrations used were 0, 100, 200 and 300 ppm. The 1000 ppm $AlCl_3$ stock was then diluted to make 100, 200 and 300 ppm of Al solution. The pH was set at 5.5 for 0 ppm (control) and ± 3.5 for Al-treated soils. The Al solutions were applied every day in the morning with a volume of 30 ml per polybag for 32 days (Supena et al. 2014).

Genomic DNA extraction, quantification and quality measurement

Total genomic DNA was obtained from each capsicum variety. Leaves of each capsicum variety were used to extract the genomic DNA (Jadid et al. 2016). The extraction was carried out using Geneaid® DNA Mini Kit according to the protocols provided by the manufacturer. The quantity and quality of total genomic DNA were determined using NanoDrop (Thermo Scientific™ nanodrop 2000). The concentration of the DNA extract was expressed as ng μl^{-1}, meanwhile, the quality of the genomic DNA extract was evaluated based on the A260/A280. Good quality of DNA extract ranges from 1.8-2.0 (Animasun et al. 2015; Jadid et al. 2018).

DNA amplification using ISSR markers

Genomic DNA obtained previously was used as template for DNA amplification using ISSR markers. A total of 5 ISSR primers were used in this study. These primers were synthesized by the IDT Integrated DNA Technologies. They were ISO2, Primer 5, Primer 9, ISSR-825, and ISSR-811 (Table 1) (Olatunji et al. 2015; Rana et al. 2013; Ibarra-Torres et al. 2015). PCR amplifications were conducted in a final volume of reaction 25 µl using GoTaq® Green Master Mix (Promega, USA). The DNA amplification program consisted of initial denaturation 95 ºC for 2 min, followed by 35 cycles of denaturation at 95 ºC for 30 s, annealing at primer specific temperature for 30 s, and extension at 72 ºC for 1 min. Final extension was done at 72 ºC for 5 min. The amplicons produced by PCR amplification using ISSR primers were then separated by electrophoresis at 100 volts on 2% gel agarose containing 1 µl ethidium bromide in 0.5x tris-boric acid EDTA buffer. Subsequently, the gel was visualized under UV transilluminator.

Table 1. Characteristics of the ISSR primers used for genotyping analysis of *Capsicum* varieties.

Primer	Short sequence	Extended sequence (5'→3') *Y* = (C,T)	Number of bases
ISO 2	$(AC)_8$ YG	ACACACACACACACACYG	19
Primer 5	$(GA)_7$ GC	GAGAGAGAGAGAGAGC	16
Primer 9	$(AG)_{10}$ T	AGAGAGAGAGAGAGAGAGAGT	21
ISSR-825	$(AC)_8$ T	ACACACACACACACACT	17
ISSR-811	$(CAC)_2$ $(AC)_4$ AAT	CACCACACACACACAAT	17

Data analysis

The study was conducted with a completely randomized design (CRD) factorial of two factors, the concentration of aluminum and plant varieties. Two morphological characters were measured in this study. It includes plant height and root length of each Capsicum variety. These parameters were analyzed using two-way Analysis of Variance (ANOVA) with a 95% confidence level.

The results of the PCR amplifications through gel electrophoresis were analyzed. Each ISSR band formed represented as independent locus and were scored as present (1) and absent (0). These data were then evaluated as a binary data matrix. The presence of monomorphic and polymorphic bands was determined. The percentage of polymorphism was calculated to measure the percentage of polymorphic alleles per primer used in this study (Monfared et al. 2018). The measurement was calculated using the following equation:

$$\text{Percentage of polymorphism} = \frac{\text{Number of polymorphic allele}}{\text{Total alleles}} \times 100\%$$

The polymorphism information content (PIC) was evaluated using the following formula:

PIC = 2Pi (1-Pi)

Where: Pi is the frequency of the presence of polymorphic bands in different ISSR primers. The calculation was done using *Gen Calc* software (https://gene-calc.pl/). Clustering was performed using UPGMA (Unweighted Pair Group Method using Arithmetic average) algorithm through the MVSP (Multi-Variate Statistical Package). The PIC was used to evaluate the genetic markers. Therefore, the PIC value was classified into three classes, including high informative marker (*PIC* > 0.60); moderate informative primer (0.3 <*PIC*<0.59); less informative primer (*PIC*<0.3) (Mateescu et al. 2005).

RESULTS AND DISCUSSION

ISSR analysis

ISSR analysis is a PCR-based technique used for evaluating genetic variability. Therefore, good quality DNA genomic (gDNA) extract is required. The results of the gDNA extraction from the four capsicum varieties are presented in table 2. The amount of the gDNA ranged from 40.63 to 72.44 ng μl^{-1}. In addition, good quality of gDNA was also obtained, according to the A260/A280 ratio, which ranged from 2.08 to 2.19 (Table 2). This was also reflected by clear and reproducible amplification profile (Figure 1). The quality of DNA extract influences DNA amplification. The absorbance ratio 260/280, ranging from 1.8 to 2.0 is commonly used to measure the quality of the DNA. The optical density at that range indicates that the DNA is free from protein and RNA contamination (Qamar et al. 2017).

After the good quality of gDNA was obtained, the four *Capsicum* varieties were evaluated using ISSR markers. In this recent study, genetic variation of four varieties of *C. annuum* var. *ciko, tanjung2, lembang1, and lingga* was evaluated using 5 ISSR primers, namely ISO2, PRIMER 5, PRIMER 9, ISSR-825, and ISSR 811 (Table 1). Some previous studies have reported the effectiveness of ISSR PCR-based method to demonstrate genetic variability among *Capsicum* species (Ibarra-Torres et al. 2014; Olatunji et al. 2019; Rana et al. 2014). A total of 108 bands were produced. They ranged from 200-1,700 bp (Figure 1). We obtained a total of 25 polymorphic bands.

Table 2. Concentration of the gDNA extracted from four *Capsicum* varieties

***Capsicum* varieties**	**gDNA (ng/µl)**	**OD 260/280nm**
Capsicum annuum var. *tanjung2*	43.40	2.19
Capsicum annuum var. *ciko*	40.63	2.10
Capsicum annuum var *lembang*1	72.44	2.08
Capsicum annuum var *lingga*	42.43	2.19

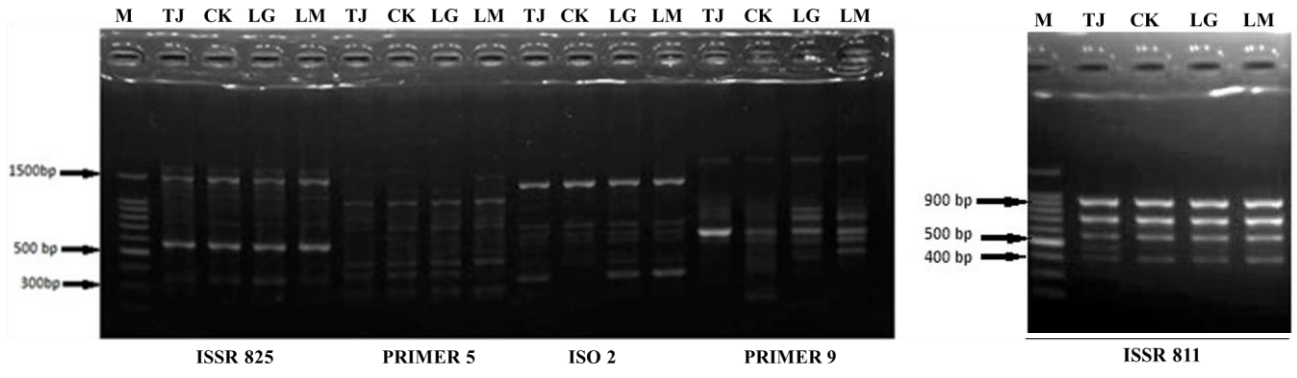

Figure 1. PCR-amplified ISSR pattern of four *Capsicum* varieties on 2% agarose gel using five marker primers. M, molecular marker; TJ, *Capsicum* var. *tanjung2*; CK, *Capsicum* var. Ciko; LG, *Capsicum* var. *lingga*; LM, *Capsicum* var. *lembang1*.

Inter-simple sequence repeats (ISSR) molecular marker is one of the DNA markers, which uses the microsatellite regions in the genome for direct DNA amplifications (Mehrotra and Goyal 2014). These markers allow us to provide the genetic information of many loci. Therefore, it can be used to monitor the genetic variability among species or interspecies populations (Sudupak 2004). Genetic diversity and genetic relatedness analysis are indispensable for crop improvement (Igwe et al. 2017). The average number of polymorphic bands and percent of polymorphism were 5 and 35.75%, respectively. Highest percentage of polymorphism (70%) was obtained by primer 9 with a total of 8 polymorphic bands, followed by primer 5 (55.56%), ISO 2 primer (33.3%), and ISSR-811 (20%). We observed no polymorphic bands in ISSR-825 (Table 3). The average percentage of polymorphism is relatively moderate. Our results are in contrast with previous study, which observed relatively low genetic diversity among other *Capsicum* varieties (Olatunji and Afolayan 2019). Our results suggest that *Capsicum* varieties from Indonesia might possess different genetic profile than others *Capsicum* varieties around the world.

The efficacy of the ISSR markers used in this study was quantified by PIC, which varied from 0 to 0.64. The average PIC value in this study was 0.29 (PIC> 0.20). This indicates their effectiveness (Mandal et al. 2013). According to Mateescu et al. (2005), the PIC value is divided into three classes, high informative marker (*PIC* > 0.60); moderate informative primer (0.3 <*PIC*<0.59); less informative primer (*PIC*<0.3). Highest PIC value was demonstrated by primer 9 (0.64), followed by primer 5 (0.39), ISSR-811 (0.19) and ISO 2 (0.15). Meanwhile, the minimum PIC value was showed by ISSR-825. Therefore, primer 9 is classified as highly informative marker and primer 3 as moderate informative marker. This is in line with previous study which stated that primer 9 is high informative marker to demonstrate the genetic variability among *Capsicum* (Olatunji and Afolayan 2019). This also indicates that primer 9 is important marker for linkage analysis (Hildebrand et al. 1992). Other ISSR markers used in this study were less informative.

Cluster analysis

Data from DNA amplification was scored and used subsequently to construct a dendrogram using UPGMA analysis to cluster the *Capsicum* varieties cluster. UPGM dendrogram was constructed based on the genetic similarity among plant species according to the molecular marker used (Dikshita and Sivarajb 2015). Our results showed that four *Capsicum* varieties were clustered into two major groups. Cluster 1 consisted of *C. annuum* var. *lembang1* and var. *lingga* at 0.5 similarity level. Whereas, the second group consisted of *C. annuum* var. *ciko* and var. *tanjung2* at similarity level of 0.6 (Figure 2).

Morphological responses of Capsicum varieties against Al stress

Observation of the morphological responses of *C. annuum* varieties against Al stress was carried out after 32 days of aluminum stress treatment (Figure 3). Our data showed that Al stress inhibited *Capsicum* growth. We observed that plant height of all *Capsicum* varieties tested in this study was reduced following an increase of Al stress level (Figure 4). *C. annuum* var. *ciko* exhibited the greatest reduction of plant height among the varieties. This is in line with previous study conducted by Won et al. (2013) which stated that giving heavy metal Al can reduce plant height. The higher the Al stress concentration given, the higher the plant height is inhibited.

The statistical analysis showed that the interaction between Al concentration and *Capsicum* varieties was not significantly influencing the plant height (p value > 0.05). However, we observed different levels of plant reduction among the tested *Capsicum* varieties. *Capsicum* var. *ciko* exhibited highest reduction of plant height compared to the control plants. They demonstrated 14.7%, 28% and 17.1% of plant height reduction compared to control after being treated with 100, 200 and 300 ppm of Al, respectively. It was followed by *Capsicum* var. *lembang1*, which performed 8.8%, 23.01% and 17.6% of plant height reduction compared to control after being treated with 100, 200, and 300 ppm of Al, respectively. Meanwhile, *Capsicum* var. *tanjung2* exhibited lowest plant height reduction compared to the control plant after Al treatment (Figure 4).

Table 3. Total number of amplified fragments and polymorphic information generated by five ISSR primers in four *Capsicum* varieties.

Primer	Tm (^{0}C)	TL	LP	TNB	NP	%P	PIC
ISO 2	54.3	6	2	22	6	33.3	0.15
Primer 5	47.0	9	5	25	10	55.56	0.39
Primer 9	52.8	10	7	20	8	70	0.64
ISSR-825	51.4	6	0	24	0	0	0
ISSR-811	52	5	1	17	1	20	0.19
Total		36	15	108	25		
Mean		7	3	20.8	5	35.77	0.29

Note: Tm (^{o}C)-Annealing temperature; TL-total loci; LP-polymorphic loci; %P-percentage polymorphism; NB-total number of bands; NP-total number of polymorphic band; PIC-polymorphic information content.

Aluminum accumulation had been reported to reduce the adsorption rate of water and nutrient, including phosphate from soils through plant root (Karimaei et al. 2016). Al^{3+} will bind the $H_2PO_4^-$anion and precipitate it as an Al-phosphate reaction through the $H_2PO_4^-$+ $2OH_2^-$+ Al_3 +-> Al (OH) $2H_2PO_4$ reaction. This causes phosphate deficiency because plants always absorb phosphate as $H_2PO_4^-$, $HPO_4^{2-}$and PO_4^{3-}which are mainly available in the soil. (Raharjo et al. 2007). In addition, a decrease in potassium and calcium nutrients was also observed in wheat that was exposed to aluminum (Al) stress (Oh et al. 2013). Calcium (Ca) is a component of plant cell walls and regulates plant cell wall construction while plants use potassium (K) to activate enzymes, photosynthesis, protein formation and sugar transport (McCauley 2011).

Our observation also showed that Al stress treatment also interfered root growth. Statistical data analysis showed that interaction between *Capsicum* varieties and Al concentration was significantly influenced root length (p-value < 0.05). The lowest average length of the main root was demonstrated in *C. annuum* var. *lingga* which Al stresses at a concentration of 100 ppm. Meanwhile, *C. annuum* var. *lembang1* showed a decrease in root length after being treated with Al stress at 100 and 300 ppm. Finally, var. *ciko* exhibited root growth inhibition when they were exposed to Al stress at 200 and 300 ppm (Table 4).

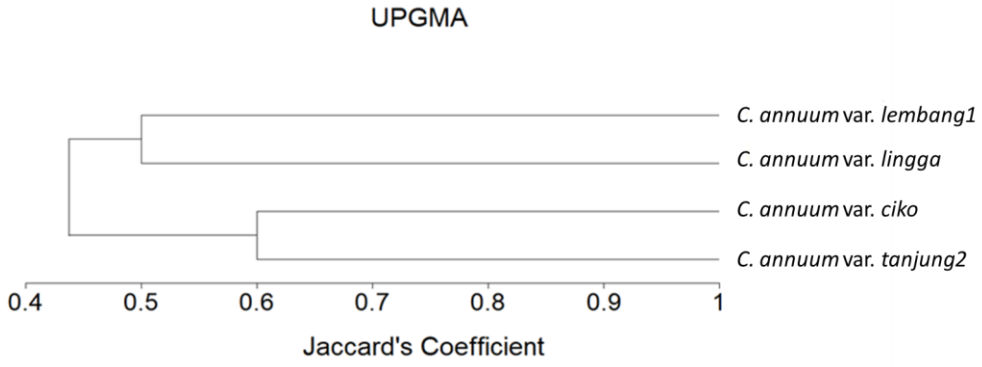

Figure 2. ISSR-based dendrogram of genetic similarity among *Capsicum* varieties.

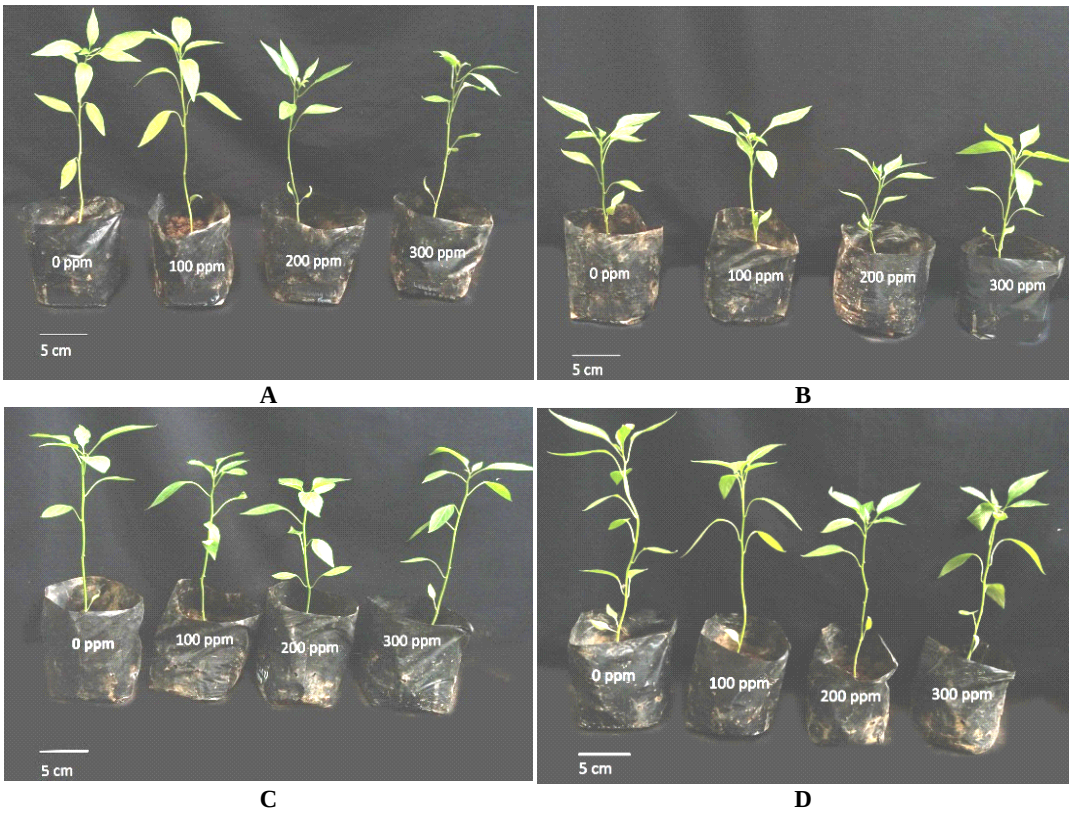

Figure 3. Observation of morphological responses of *Capsicum* varieties against Al stress treatment at 73 days after planting (DAP). A. *Capsicum annum* var. *lembang1*, B. *Capsicum annum* var. *ciko*, C. *Capsicum annum* var. *tanjung2*, D. *Capsicum annum* var. *lingga*

Table 4. Response of *Capsicum* varieties on root growth under Al stress treatment at 73 days after planting (DAP).

Al conc. (ppm)	Root length (cm)			
	Lingga	**Lembang1**	**Tanjung2**	**Ciko**
0	3.46 ± 1.03 ^{ab}	2.34 ± 0.23 ^{bc}	2.36 ±0.13 ^{bc}	1.8 ± 0.57 ^{c}
100	1.6 ± 0.38 ^{c}	2.3 ± 0.63 ^{abc}	2.62 ± 0.4 ^{abc}	2.22 ± 0.41 ^{c}
200	2.6 ± 1.47 ^{abc}	3.04 ± 0.42 ^{abc}	4.02 ± 0.62 ^{a}	1.74 ± 0.4 ^{c}
300	1.64 ± 0.61 ^{c}	2.14 ± 0.22 ^{bc}	2.2 ± 1.15 ^{bc}	1.6 ± 0.49 ^{c}

Note: the numbers with the same notation in the Tukey test are not significantly different (P<0.05)

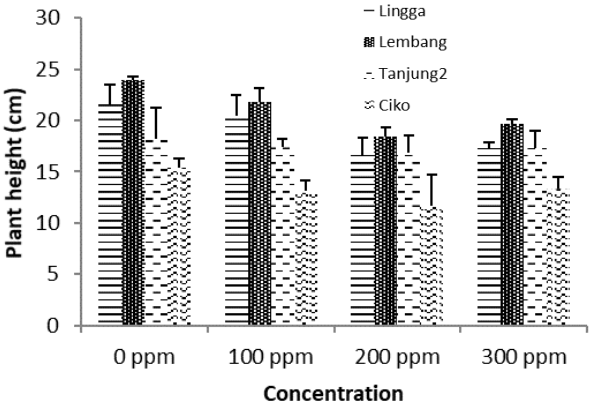

Figure 4. Response of *Capsicum* varieties on plant height under Al stress treatment at 73 days after planting (DAP)

Plants have developed different mechanisms to cope with heavy metal stress (Jadid et al. 2017; Manara, 2012). Heavy metal stress might induce program cell death (PCD). Consequently, some plant organ growth will be inhibited (Huang et al. 2014). Another study also demonstrated that AlCl3 inhibited the growth of peanut roots along with the increase of Al stress concentration and exposure time. It had been also described that Al stress-induced reactive oxygen species (ROS) production along with the increase in Al exposure. Therefore, PCD might be a consequence of massive ROS production within plant cells (Huang et al. 2014). The formation of ROS had been reported occurred in protoplast after 4h of Al exposure. This free radical is generated from the mitochondria and it increases along with the Al induction (Huang et al. 2014).

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