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Morphological characteristics and isozyme banding patterns of *Cucurbita moschata* at different altitudes

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Abstract. *Hidayati NR, Suranto, Sajidan. 2018. Morphological characteristics and isozyme banding patterns of* Cucurbita moschata *at different altitudes. Biodiversitas 19: 1683-1689.* Aims of this research were to investigate the morphological character and isozyme banding patterns of *Cucurbita moschata* plants grown at three different altitudes. Samples in this study consisted of leaf, stem, and flowers. The morphological characters were conducted by direct observation in the field and analyzed descriptively as well as statically by one way ANOVA. The isozyme bands appearance of esterase and peroxidase of leaf samples were conducted using polyacrylamide gel electrophoresis (PAGE). Qualitative approach was used to analyze the presence and the absence of isozyme bands, while Retardation factor (Rf) was used to analyze quantitatively. The results showed that most plants grown at middle altitude (351-750 m asl.) were well-developed in terms of length of leaves, stems and flowers. Accordingly, the isozyme banding pattern of peroxidase was also found varied in plants grown at middle altitudes from which the presence of very unique bands was detected. Conversely, the band detected in plants grown at the lower and the highest altitudes was similar in term of band's number but it was different in the quality of the bands. Meanwhile, esterase isozyme banding pattern of plants grown at the lower and higher altitude had more bands than the middle altitude. Based on this result it is obvious that the isozyme data could be used to support in understanding the diversity morphological characters of plants grown in three different altitudes. This early result suggests that altitudes as a crucial factor in contributing the expression of isozyme appearance, which is useful for further pumpkin characterizations.

Keywords: altitude, *Cucurbita moschata*, isozyme, morphology

INTRODUCTION

Cucurbita moschata D., which belonged to the family of Cucurbitaceae, have been recorded its ability to grow and develop in both the tropical area and the sub-tropic region with altitudes of 2200 m asl. (Paris 2010; Jacobo-Valenzuela et al. 2011). As perennial plants, pumpkin fruit is a valuable source of energy and nutrition due to contents of carbohydrate, lipids, protein, and minerals are high (OECD 2012; Suranto et al. 2015). Besides, its fruit contains sugar such as fructose, glucose, sucrose, myo-inositol and raffinose (Kami et al. 2011), this fruit also produces secondary metabolism, i.e., α and β carotenes acting as an antioxidant (Zaccari and Galetta 2015). This plant is easy to grow worldwide including in the area of Tegal District of Central Java-Indonesia. The altitudes of this district were ranging from lower land area or even 100 meters below sea level until more than 1200 meters above sea level (m asl.) (BPS 2017). These habitats enable plants to grow and adapt to the varied environmental conditions. The morphological variations of pumpkin plants could be interpreted as the active response of plant organs to varied environmental factors, such as soil, temperatures and weather conditions. These environmental factors could cause the morphological appearances of plant varied in between their populations, and this occurrence could be observed both during vegetative and generative periods. Many approaches have been employed in order to know the diverse morphological appearance was due to genetically induced variation, or environmentally induced variation. In recent years, the characterization of plants has been conducted not only based on morphological characters but also the isozyme banding pattern as reported by Premoli (2003) and Zolfghari et al. (2010). The use of plant isozyme in the characterization of plants have been conducted widely (Rejon et al. 2012; Houmani et al. 2016; Hartanti et al. 2017).

The electrophoretic isozyme approach was not only used to clarify the position of plant species in the correct taxon, but it could also be used to detect the presence of certain disease on plant organs (Suranto et al. 2017). As reported by Gautam et al. (2018) that isozyme banding pattern could be used to determine the effect of heavy metal in the soil on particular crop. Isozyme banding pattern has been widely used by researchers because of the electrophoretic plant proteins can be conducted very easily. And therefore esterase and peroxidase were chosen as the first consideration due to the fact that only small amount of samples used and only very limited times required as well as very low prices needed in running the experiment. Therefore in this study, morphological characters and isozyme banding pattern data were conducted in order to investigate the differences of *C. moschata* grown at three different altitudes.

MATERIALS AND METHODS

Environmental measurement

Prior to collecting the data for laboratory works, a number of environmental parameters were tested in the field. There was relative humidity (%), temperature **(°**C), soil pH and the quality of light intensity. All data were collected at three different level of altitudes.

Samples locations

Pumpkin plants used in this study was local rounded fruits type which is collected in every altitudes location of Tegal District, Central Java, Indonesia. The stratified sampling method was employed, and the only flowering plants were taken as a sample. There were three different altitudes used for sampling locations: I (1-350 m asl.), II (351-750 m asl.), and III (751-1050 m asl.). Those sample locations were classified as lower (I), middle (II), and high (III) altitudes, respectively. Observation in every single location of sampling was observed three times (Figure 1).

Morphological character

Stem

Thirty-six of stems from every single plant-resulted from every three different altitudes were used to characterize the stem morphology. The third internode from the top was chosen as the sample, which was then measured by its length, diameter, shape, surface, color, and hairness of the stem. The length and width were calculated quantitatively, while for the rest data were analyzed qualitatively.

Leaf

Using the same number as stem samples, leaves were measured in terms of length, width, and diameter of leaf, length, and diameter of petiole, petiole stripe, shape, dentateness, apex, basal, venatio, color, nervus lateralis, as well as hairness.

Flower

Employing thirty-six of flowers collected from every single plant at three different levels of altitudes. The flower characters such as color, number of petals, diameter and length of flowers, pedicellus, length and total number of calyxes.

Planting procedures

The transplanted plants from the natural habitat were collected and planted in the experiment field at Departement of Biology, UNS. Forty-five (45) plant samples from nine (9) locations of three (3) different altitudes were grown in 45 polybags containing compost soil. Watering was done every two days using tap water, and fertilizer was given once in three weeks times.

Electrophoresis and isozyme procedures

Total number of 45 leaves were collected from three different location (Figure 1). Procedures of running electrophoresis of isozymes which consisted of preparation of solution, acrylamide gel, extraction of sample, electrophoresis, and staining procedure were conducted according to Suranto (2001), with some modification.

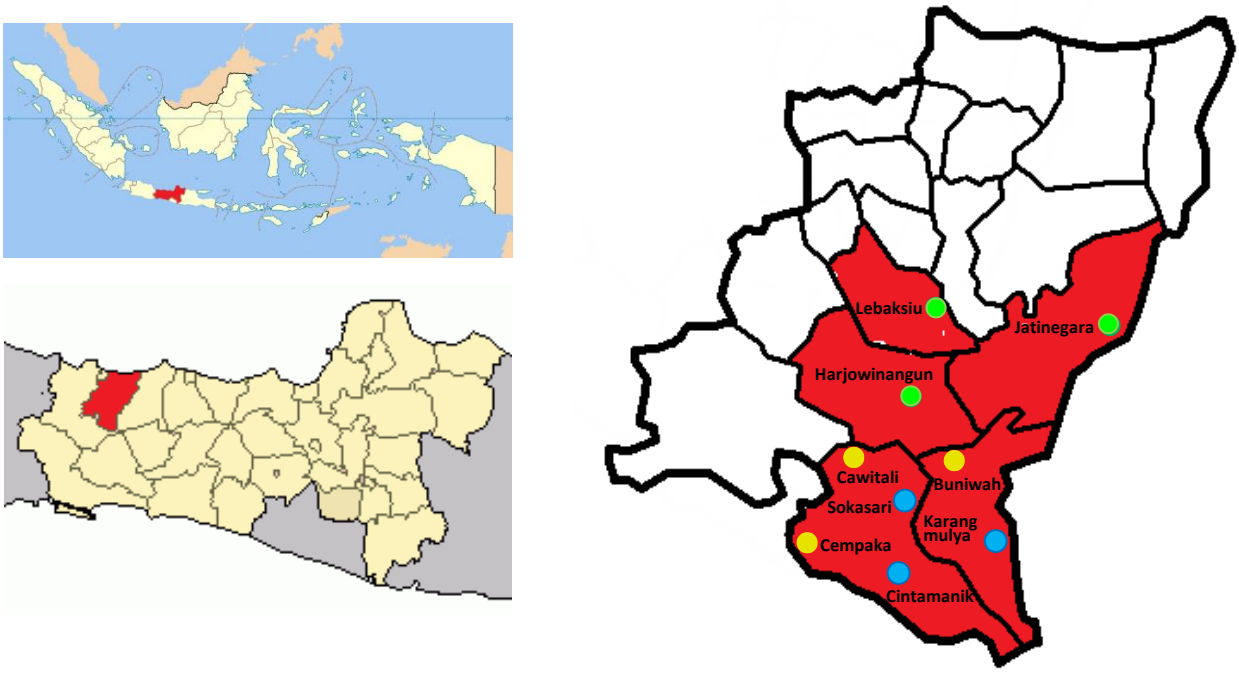

Figure 1. Map area of Tegal District, Central Java, Indonesia, where samples of *Cucurbita moschata* are collected. Note: ● = 1-350 m asl., ● =351-750 m asl., ● = 751-1050 m asl.

Buffer solution

Buffer solution consists of a tank buffer that is used during the electrophoresis process and extraction buffer which is used to extract fresh leaves. The buffer tank is made by dissolving 14.4 grams of boric acid and 31.5 grams of boric into distilled water until it reaches a volume of 2 liters. Extraction buffer is made by dissolving 0.018 grams of cysteine, 0.021 grams of ascorbic acid, and 5 grams of sucrose into 20 ml of pH 8.4 tank buffer.

Stock solution

Stock solutions consist of stock A made by dissolving 4.5 grams of TRIS (Hydroxymethyl) Methylamine (PURISS); 0.51 grams of citric acid, and 500 ml of distilled water and stock B by dissolving 30 grams of acrylamide; 0.80 gram N N'-methylene-Bis-Acrylamide; and 100 ml of distilled water. Stock A and stock B are prepared for making of acrylamide gel. *Preparation of acrylamide gel* Acrylamide gel is made by mixing 5 ml of stock A solution and 2 ml of stock B solution then being shaken until homogeneous. After homogeneous 5 µl TEMED is added and 7.5 µl 10% APS.

Leaf extraction A total of 0.5 g of fresh leaves were extracted using 0.5 ml of extraction buffer. The leaves are mashed using cold mortar to maintain the stability of the enzyme. Leaf samples were centrifuged at a speed of 4000 rpm for 3 minutes. The supernatant obtained is used for the electrophoresis process.

Electrophoresis

Peroxidase: as much as 2.5 µl of supernatant were inserted into the gel well and run at constant voltage (80 V) for 55 minutes. Esterase: as much as 3 µl of supernatant were put into a gel well and run at a constant voltage (80 V) for 55 minutes.

Enzyme staining

Peroxidase: the gel is soaked into the peroxidase enzyme dye made by dissolving 0.0125 grams of O-Dianisidine into 2.5 ml of acetone. Next, 20 ml of acetate buffer pH 4.5 was added and 2 drops of H_2O_2 were added. The gel was soaked for 5 minutes until the band pattern appeared and rinsed using distilled water.

Esterase: esterase enzyme staining was carried out by mixing 0.025 grams of 1-naphthyl acetate, 0.0125 grams of fast blue BB salt dissolved in 2.5 ml of acetone and 20 ml of phosphate buffer pH 6.5. Gel is left for 3 hours and rinsed with distilled water

Data analysis

For the morphological data have been analyzed descriptively, meanwhile, the morphometric data was analyzed using One way ANOVA, and then followed by Duncan Multiple Range Test (DMRT). Data of electrophoretic isozyme were analyzed both using qualitative and quantitative methods. For the presence and absence of the bands detected including the thickness. Qualitative approach was chosen, meanwhile, the movement of bands was calculated based on the Retardation factor (Rf) as explained by Lehmann et al. (1989).

RESULTS AND DISCUSSION

Environmental conditions

The condition of microclimate of each altitude is presented at Table 1. All parameters of environmental conditions varied except for the soil pH (7). In this study, air and soil temperatures decreased along with the increased altitudes. Meanwhile, contrary pictures were recorded for the relative humidity (RH), and no different data were obtained when soil pH from three altitudes was tested. The lowest light intensity was found at the highest altitude, while the highest light intensity recorded in the low altitude.

Morphological characters

Pumpkin plants grew very well at the second altitudes (Table 2). This could be proved by the result of the stem length (12.567±0.071 cm) and stem diameter (1.023±0.008 cm). For the rest of two altitudes showed quite similar in their length and diameter. In general, the shape of observed stem was similar (pentagonal). However for the color of stem and hairness were a bit varied. Dark green was detected for the plants which grew at second altitudes (351-750 m asl.), while for the other two altitudes were light green. Accordingly, the very dense, smooth, long hairness were detected at the highest altitudes, while for the lowest altitude occurred conversely. In all cases, the second altitudes of plants showed to have quite long dense hairs with the texture was quite rough.

For the leaf character examinations, there was significant difference on the length of leaf and petiole, but those in the first (I) and second (II) altitudes were not examined. It is recorded that the longest leaf was found at second altitude (12.966±0.055 cm). Conversely, the longest petiole was found at the first altitude (9.100±0.055 cm) and the smallest of petiole diameter (0.426±0.005 cm) was detected at the first altitude. This occurrence was also recorded for narrowest leaf (10.800±0.550 cm) which was found at the first altitude.

The morphological characters of leaves were examined for their shape, dentateness, basal leaf, hairness, apex, venatio, and nervus lateralis. Results showed that the morphological characters of leaves were indifferent in leaf, shape, dentateness, and basal (Table 5). However, the color of leaves, as well as the hairness, were quite varied. The light green was detected at the highest altitude.

Other morphological characters such as flower were also shown similarly. The only petiole and calyx length of *C. moschata* flowers were recorded varied, but the other characters were not (Table 6).

Table 1. Microclimate condition of each three different altitudes

Parameters ($\bar{X}$)	Altitudes (m asl.)		
	I (1-350)	II (351-750)	III (751-1050)
Air temperature (°C)	38.64	33.82	26.95
Relative humidity (%)	53.55	56.22	81.00
Soil temperature (°C)	31.06	28.41	25.33
Soil pH	7	7	7
Quality of light intensity	High	Normal	Low

Table 2. Result of morphological character tested of *Cucurbita moschata* a three different level of altitudes

Stem characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Length	9.800[a]±0.057	12.567[b]±0.071	11.733[c]±0.082
Diameter	0.690[a]±0.005	1.023[b]±0.008	0.786[c]±0.007

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

Table 3. Result of morphological characters tested from *Cucurbita moschata* at three different level of altitudes

Stem characters	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Shape	Pentagonal	Pentagonal	Pentagonal
Colour	Light green	Dark green	Light green
Stripe surface	Clear	Very clear	Clear
Hairness	Rough, short, very seldom	Pretty rough, long, Pretty dense	Smooth, long, very dense

Table 4. Result of leaf character tested of *Cucurbita moschata* at three different level of altitudes

Leaf characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Length	11.833[a]±0.088	12.966[b]±0.055	11.867[c]±0.149
Width	10.800[a]±0.550	11.633[b] ±0.080	11.867[c]±0.054
Diameter	11.267[a]±0.083	12.200[b]±0.119	11.867[ab]±0.149
Petiole diameter	0.426[a]±0.005	0.517[b]±0.005	0.623[c]±0.005
Petiole length	9.100[a]±0.055	7.883[b]±0.108	6.850[c]±0.098
Total of petiole stripe	13.000[a]±0.301	12.667[a]±0.333	12.083[a]±0.358

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

Table 5. Leaf morphology of *Cucurbita moschata* observed at three different level altitudes

Leaf characters	Altitude (m asl.) I (1-350)	II (351-750)	III (751-1050)
Shape	Orbicularis	Orbicularis	Orbicularis
Apex	Acuminatus	Acuminatus	Acuminatus
Dentateness	Serratus	Serratus	Serratus
Basal	Emarginate	Emarginate	Emarginate
Venatio	Palminervis	Palminervis	Palminervis
Colour	Dark green	Dark green	Light green
Nervus lateralis	Very clear	Clear	Clear
Hairness	Smooth, dense	Pretty rough, pretty dense	Rough, very dense

Table 6. Flower characters *Cucurbita moschata* examined at three different level of altitudes

Flower characters (cm)	Altitudes (m asl.) I (1-350)	II (351-750)	III (751-1050)
Pedicellus length	8.433[a]±0.068	15.933[b] ±0.068	4.600[c]±0.086
Diameter	13.308[a]±0.096	13.600[b]±0.082	10.067[c]±0.08
Calyx length	5.083[a]±0.078	4.167[b]±0.061	3.608[c]±0.104
Length of petal	9.225[b]±0.056	9.267[b]±0.072	7.100[a]±0.070

Note: Based on the Duncan Multiple Range Test (DMRT), the numbers followed by the different letters in same row are significantly different, P<0.05

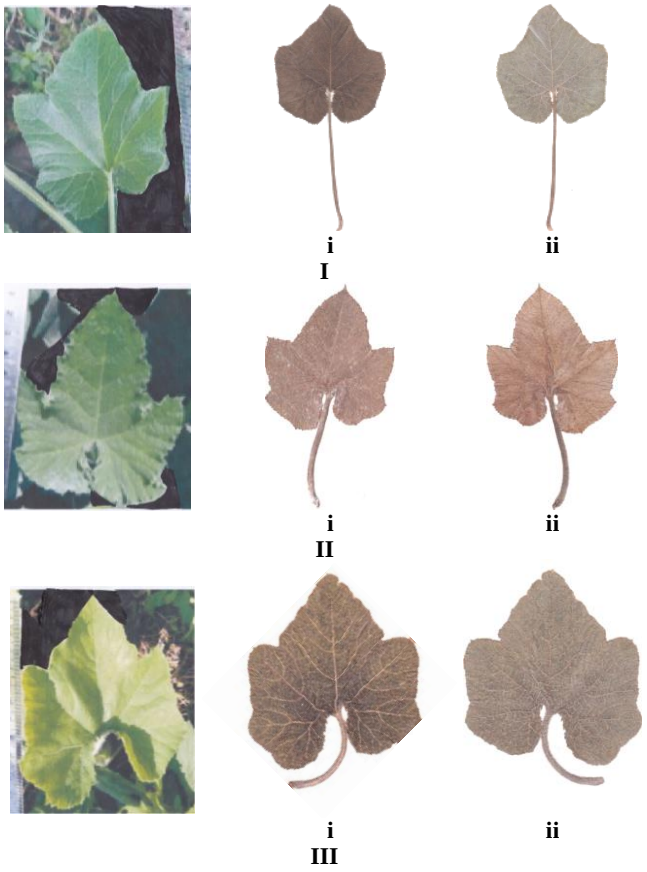

Figure 2. Morphological character of *Cucurbita moschata* leaves taken from three different levels of altitudes. Note: I (1-350 m asl.), II (351-750 m asl.), III (751-1050); (i) upper surface of leaves, (ii) lower surface leaves

Figure 3. Flower character of *Cucurbita moschata* at different level of altitudes. Note: I (1-350 m asl.), II (351-750 m asl.), III (751-1050 m asl.)

Table 7. Flower characters *Cucurbita moschata* examined at three different level of altitudes

Flower character	Altitude (m asl.)		
	I (1-350)	II (351-750)	III (751-1050)
Colour	Yellow	Yellow	Yellow to violet
Total number of calyx	5	5	5
Total number of petal	5	5	5

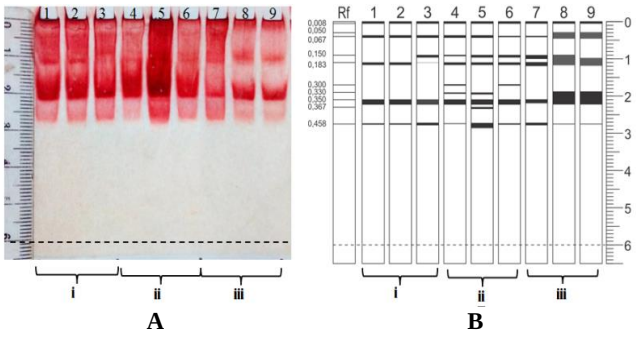

Figure 4. Peroxidase isozyme of leaf samples of *Cucurbita moschata* collected from three different lands of altitudes. Note: 1. 130 m asl., 2. 267 m asl., 3. 330 m asl., 4. 411 m asl., 5. 560 m asl., 6. 615 m asl., 7. 756 m asl., 8. 880 m asl., 9. 962 m asl. i. lower, ii. middle, iii. high. A. Band pattern, B. Zymogram

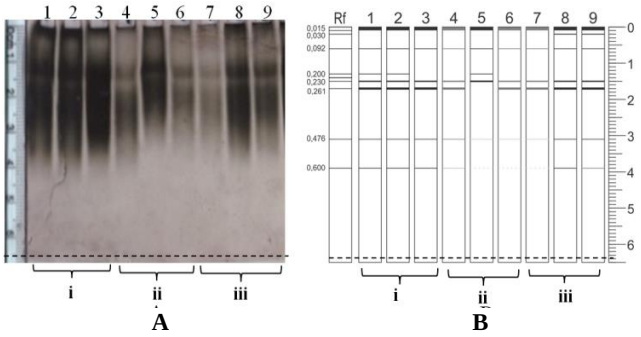

Figure 5. Esterase isozyme of leaf samples of *Cucurbita moschata* collected from three different lands of altitudes. Note: 1. 130 m asl., 2. 267 m asl., 3. 330 m asl., 4. 411 m asl., 5. 560 m asl., 6. 615 m asl., 7. 756 m asl., 8. 880 m asl., 9. 962 m asl. i. lower, ii. middle, iii. high. A. Band pattern, B. Zymogram

The longest petiole was found at the second altitude (15.933 ±0.068 cm). In this altitude, the widest of diameter of flower (13.600±0.082 cm) was also found. Meanwhile, the longest calyx length was recorded at the first altitude (5.083±0.078 cm), and the shortest was noted at the highest altitude (3.608±0.104 cm). It is interesting to note that the flower color of this pumpkin plant turns out to be violet (yellow to violet) when grown at the highest altitude. But the other characteristics were found no different (Table 7).

Isozyme analysis

The results of peroxidase isozymes analysis show differences in the banding pattern formed at three altitudes (Figure 4). The Rf values formed to range from 0.008 to 0.458. As many as 10 Rf, that was 0.008, 0.050, 0.067, 0.150, 0.183, 0.300, 0.330, 0.350, 0.367, 0.458 formed from a range of 1-350 m altitude to 751-1050 m asl. Rf formed at lowest altitudes (1-350 m asl.) and highest (751-1050 m asl.) altitudes had a similar pattern of band appearance with the value of Rf 0.008, 0.067, 0.183, 0.350, and 0.458. However, the thickness of the band at high altitudes thicker than the low altitude. There is one additional Rf, which is at Rf 0.150 at 330 m asl. and 756 m asl. Bands formed in the middle altitude are the most varied, i.e., the formed bands increase at Rf 0.300 (411 m asl. and 615 m asl.), 0.330 (411 m asl., and 560 m asl.), and Rf 0.367 (560 m asl.). The pattern of esterase isozyme bands (Figure 5) formed less varied than that of the isozyme peroxidase (Figure 4).

The bands produced by esterase isozyme were 8 (eight) bands, ranging from 0.015 to 0.600, such as Rf 0.015, 0.030, 0.092, 0.200, 0.230, 0.261, 0.476, and 0.600. Two additional bands were recorded at lower altitude (0.200 for the location of isozyme 130 m asl. and 267 m asl.). There was no band pattern obtained from esterase leaf samples in the middle altitude especially 560 m asl. at Rf 0.0261. However, the band at Rf 0.200 was detected. Based on the thickness band from esterase samples of the middle altitude, it showed that band at 0.600 was very painful. At the highest altitude, particularly at 880 m asl. and 960 m asl, the presence of band was only at Rf 0.030.

Discussion

The axis level of altitude locations will produce different light intensity. The highest altitude would almost always produce low level of light intensity. Therefore, the temperature will also very low and relative humidity would eventually go up. The lower temperature will influence the lowest of soil temperature. Other environmental factors such as oxygen level, soil condition, type of soil, as well as soil porosity, would contribute to the plant morphological appearance (Yuliani et al. 2015). Those all environmental factor would produce different morphological characteristics such as stem, leaf, flower both quantitatively and qualitatively. It is generally accepted that plants would adapt to the environmental condition both physiology and morphology (Gong et al. 2018) and it is recorded that plasticity of plant has been recorded as a good way of plant in responding the different environmental conditions (Frei et al. 2014). In addition, the plant plasticity of stem will be influenced by light intensity (Ye at al. 2017). This phenomenon has been shown by the length and diameter of stem particularly at middle altitude as compared to other altitudes. Yuliani et al. (2015) reported that plant in the family Asteraceae possessed more stem length at middle altitudes compared to the lower and highest altitudes. This occurrence was confirmed by Kofidis et al. (2007) in which the plant would grow maximally under favorable conditions and would not depend on the highest or lowest habitat.

In general, the color of stem of *C. moschata* particularly grew at middle altitudes was darker compared two other altitudes. This could be due to the optimum of light intensity produced, and this could influence the anthocyanine pigment on the stem, so that it will influence the quality of stem color (Cruz et al. 2012).

Plant growing at lower altitude usually has a narrower leaves as compared to middle and highest altitudes. Pan et al. (2013) recorded that the leaf shape of plants growing at highest altitude is normally bigger. This might interpret as a result of limited nutrient availability and this resulted in very slowly transportation, and this wider leaves needed to catch the sun in order to fulfill the plant nutrition. This condition will also influence the plant petiole. In this condition usually, plants have also very small petiole. In addition, plant grow at the highest altitude usually have leaf color younger than the other altitudes. It is noted that the quality of light intensity at higher altitude (III) could not be maximized of plants for their petiole development due to the big leaf diameter. Good quality of light intensity would result in producing the content of chlorophyll A and B. This chlorophyll would derivate the plant photosynthesis, and this would eventually influence the growth of plant normally (Samuoliene et al. 2007; Abidi 2012).

Morphological character particularly the length and diameter of flower at middle altitude gave the highest influence on plant metabolism especially carbohydrate in the leaf during reproduction phase (Bhandari et al. 2016). Besides, plant has ability to response the lower or higher temperature changes by remodeling lipid membrane and defencing unsaturated lipid or change both lipid membrane or unsaturated (Zheng et al. 2011). The smallest value of pedicels length and flower diameter was recorded at the highest altitude. Yaqoob and Nawchoo (2015) noted that length and diameter of pedicels tended to be smaller at the high altitude. This could cause the plant flowering earlier at lower altitude (Frei et al. 2014). The length of petal at the lower and middle altitude significantly different with the highest altitude. Dierig et al. (2006) reported that flowering time and the change of air temperature at lower altitude influence the growth and development of plant.

The flower colors at the highest altitude usually look brighter when they were compared with other altitudes. This occurrence may be due to different light intensity could influence the distribution of color pigment on the plant (Cruz et al. 2012). Moreover, Shrestha et al. (2014) said the difference in flower color could be influenced by pollinator richness within a plant habitat. The morphological diversity of leaf, stem, and flower in three different level of altitudes may need further evidence such as electrophoretic isozyme to make sure whether this could be genetically induced (Lo Sciavo et al. 1983).

The use of isozyme banding pattern has been used to differentiate the variation of two type of *C. moschata* (Wu and Cao 2008). Varied band was recorded to lower altitude to the higher altitude. The highest total number of bands were recorded at middle altitude rather than lowest or highest altitude. This peroxidase which is included into oxidoreductase enzyme could act as an antioxidant on the plant (Rothe 1994). This occurrence evidence confirmed that middle altitude has resulted in the optimization of enzyme peroxidase metabolism.

Gulen and Eris (2014) noted that sensitivity of peroxidase would be influenced by the difference of temperature. Different altitude with the alteration of air temperature influences the appearance of peroxidase banding pattern. This occurrence could be observed from the appearance of peroxidase isozymes in both of these altitudes in which showing thicker bands compared to the middle area (Rf 0.0183 and 0.350 at Figure 4).

The isozyme banding pattern of esterase showed its high activity at both lowest and highest altitudes compared to the middle one. This activity was quite different as the peroxidase did. This phenomenon may indicate that every single enzyme would response different to the different environmental condition (Ivachenko et al. 2016).

Subramani et al. (2011) recorded that this esterase, as a hydrolytic enzyme which has function in the seed germination and plant maturation. Thus, at both altitudes of highest and lowest, plant adapt to the unfavorable conditions and this could be expressed by more detected in additional bands or was thicker bands (Figure 5).

Based on the morphological appearance of leaf, stem, and flower as well as isozymes banding pattern, *C. moschata* tended to be better growing at middle altitude, rather than other altitudes. The warm temperature would eventually enhance the growth and development of the plant. And this three different altitudes could be used as on early evidence in using the environmental factor in characterizing the pumpkin plant.

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