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Study on the growth traits in eighth-month beans (*Vigna unguiculata* L.)

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Abstract

Eighth-month bean is a variant form of cowpea [*Vigna unguiculata* (L.) Walp] and is an important component in sacrifice ceremonies in Taiwanese Plains Aboriginal culture. Among the different colors of eighth-month bean, purplish pods contain a relatively high content of anthocyanins and are rich in nutrients. However, the seed phenotype of eighth-month bean varies, and currently, little is known about the association of seeds with plant phenotypes and that of plant phenotypes with pod colors. This study distinguished the external appearance of seeds into three different types: brown seeds, seeds with variegated markings, and black seeds; this study also investigated the leaves, flower color, flowering time, pod color, pod length, weight of single pods, and number of seeds in plants grown from the different types of seeds. The results showed that plants grown from brown seeds had deep-green leaves with obvious leaf veins, light-purple flowers, and seed pods with six different colors (deep purple, light purple, purplish green, white, light green, and deep green). Among these, light-green and deep-green seed pods were the longest at approximately 29 cm, while white pods were the shortest at approximately 21 cm. The heaviest single pod weight (17.5 g) and the largest average number of seeds (16.7) were found in deep-green pods. Plants grown from seeds with variegated markings had light-green leaves, deep-purple flowers, and light-green and light-purple pods. The light-green pods were the longest (29.7 cm), heaviest (16.2 g), and with the most number of seeds (17.3). Plants grown from black seeds had light-green leaves with non-obvious veins, white flowers, and deep-green seeds and had a pod length of 30.6 cm, a pod weight of 15.2 g, and an average number of 18.7 seeds. The test for determining the anthocyanin content showed that anthocyanin was undetectable in plants with green leaves or green pods and that it could only be detected in plants with purple or white pods.

Keywords:*Eighth-month bean, Growth investigation, Agronomic trait, Anthocyanins*

1. Introduction

String beans (*Phaseolus vulgaris* L.) and cowpeas (*Vigna unguiculata*) are important food sources in the tropical and subtropical regions. At the same time, leguminous crops have important contributions to food security, economy, and soil fertility (Beebe, 2012; Ehlers and Hall, 1997). “Eighth-month beans” (Vigna unguiculata (Linn.) Walp) and cowpeas belong to the same genus and originated from Africa. In early era of Taiwan, eighth-month beans were cultivated in the Laonong Community of Liugui District in Kaohsiung by the Taiwanese plains aborigines. Among these beans, black eighth-month beans are made into “douzaimimai (bean mixed rice ball)”, which is an important dish for sacrifices. Eighth-month beans are harvested during the eighth and ninth lunar months, and are known as “eighth-month beans” since more harvest can be made during the eighth month. Eighth-month beans prefer to grow

in warm climates with abundant sunshine, and have characteristics such as drought tolerance, moisture tolerance and heat tolerance. The optimal temperature for seed germination is 20–30℃, and the optimal growth temperature is 25–35℃. In Taiwan, the fifth to the seventh lunar month is suitable for planting, and harvesting is carried out from the eighth to the tenth lunar month. Eighth-month beans are vines, and support must be erected during the early growth phase for vines to climb and grow. Typically, eighth-month beans will flower 30-40 days after sowing and can then be harvested when the pods are full. Eighth-month beans have pinnately trifoliate leaves and papilionaceous flowers that can be white, dark purple or light purple. The pods formed are cylindrical in shape and immature pods begin to sag on formation. When these pods mature, their external appearance can be many different colors, including dark purple, light purple, purplish-green, white, dark green, and light green. When the seeds in the pods mature, these seeds can also be many different colors, such as brown, black, or contain variegated markings, etc.

Beans with a high starch content such as cowpeas（*Vigna unguiculata*）are the earliest foodstuffs to be viewed as having medicinal uses and a source of nutrition in agriculture (Phillips and McWatters, 1991). The pods and seeds of eighth-month beans are the main parts of the plant consumed as food, as pods contain 20-30% protein on top of having high starch content, while containing nutrients such as dietary fibers, vitamins, and folic acid (Phillips *et al*., 2003). Purple eighth-month beans also contain anthocyanins that have anti-oxidant properties (Cabrita *et al*., 2000). Hence, the nutritional value of eighth-month beans is increasingly valued.

During harvest, the pod colors of eighth-month beans are complex and the external appearance of seeds also exhibit diversity. Currently, it is unclear what plant morphology would differently shaped seeds grow into. Also, it is not known what pod color would plants with different traits produce. This results in an inability to produce anthocyanin-containing purple pods, or black beans that are used for making “bean mixed rice ball”. Therefore, this study distinguishes between seeds with three different external appearances: brown, mixed color with variegated markings, and black. After planting, the leaves, flower color, flowering time, pod color, pod length, single pod weight, and number of seeds in a single pod from plants grown from different seeds were examined. This was done in order to understand the growth characteristics of different eighth-month beans, and to provide comprehensive growth information for future production and management of eighth-month beans.

2. Materials and Methods

2.1 Crop materials

The experimental material is provided for Kaohsiung District Agricultural Research and Extension Station. According to the appearance of color in Eighth-month beans can be distinguished into: brown, variegated markings and black. The plug seedling is adopted at the initial stage of Eighth-month beans growth, with a cultivation medium made by mixing carbon fiber rice hull, coconut fiber, and peat soil in the proportion of 1:1:2. When the maize has grown for one week and the second leaf has expanded, it is transplanted to the field.

2.2 Cultivation management

In this study, each treatment is repeated twice times, there are six grow plots, and the Completely Randomized Design (CRD) method is adopted. The area of each plot is 2.5 m×1 m, there are one lines of plants in each plot, and plant spacing is 20 cm. The chemical fertilizer application is based on N:P:K in the proportion of 15 :15 :15 for kg/ha, where 2/3 is used as basal fertilizer, and the remaining is used as additional fertilizer. The first additional fertilizer is applied during cultivation and banking. The second additional fertilizer is applied when the eighth-month beans comes into flowering. Eighth-month beans support must be erected during the early growth phase for vines to climb and grow. During Eighth-month bean growth period, potassium phosphite, neem oil , wood vinegar and other organic agents are applied in order to insect pest control .

2.3 Survey items

In this experiment, agronomic phenotype of the plants grown by brown, variegated and black seeds were investigated during the growth period, the investigation including leaf traits, leaf color, flowering time, flower traits ,flower color, pod time, fruit pods traits, pod color, seeds color of progeny and germination rate of progeny seeds. In addition, the anthocyanin content of pod was measured when the pods were fully mature.

2.4 Determination of anthocyanin content

Method of determination anthocyanin content was according to Mancinelli et.al., (1975)。Take 200 mg of leaves or pods in a mortar, add some liquid nitrogen for quick grinding, and then add 80% methanol 2 mL to grinding and extraction. Get the

extract and put in a centrifuge tube， after that centrifuged at 18,000 rpm for 5 minutes in room temperature. After centrifugation, get the supernatant liquid and placed in a spectrophotometer. The content of anthocyanins was measured at 530 nm and 657 nm.

Formula of calculating anthocyanin content (μg / mg)

$$= A_{530} - 0.333\ A_{657}\ (\mu g / mg)$$

2.5 Statistical analysis

All the measurement were evaluated for significance by an analysis of variance (ANOVA) followed by the least significant difference (LSD) test.

3. Results

Eighth-month beans that are brown, black, or with variegated markings were planted in this experiment (Fig 1). During growth and development, the leaf shape, flowering time, flower shape, pod production time, pod shape, and pod anthocyanin content of plants grown from different seeds were compared.

3.1 Comparison of leaf traits of eighth-month beans

At Week 3, healthy leaves were removed for comparison. Results showed that there were no significant differences in leaf shape from plants grown from different colored seeds (Fig 2). However, leaves from plants grown from brown seeds are darker green in color compared with the other two seeds (Fig 2a), and black seeds produced leaves with the lightest coloration (Fig 2c). Observation of leaf veins and degree of wrinkling showed that brown seeds produced the deepest leaf veins, followed by seeds with variegated markings and black seeds have the shallowest leaf veins (Fig 2a,c). From the above plant traits, we can see that black seeds produced leaves which are light-green and have the most shallow leaf veins and degree of wrinkling.

3.2 Comparison of flower traits of eighth-month beans

Eighth-month beans begin to flower at Week 5, and seeds with variegated markings produce plants that have the earliest flowering time, with a mean flowering time of 39.6 days, followed by brown seeds which produced plants that flower at 39.5 days, and black seeds which produced plants that flower at 40.4 days (Table 1). However, there were no significant differences in flowering time between these three types of seeds. The flowers produced by these three different seeds were observed to have significantly different colors (Fig 3). Plants grown from black seeds produced white flowers (Fig 3c), and brown colored seeds and seeds with variegated markings produced plants with purple flowers. Among the floral organs from plants grown from seeds with variegated markings, the color of the banner and the wing are dark purple in color, and the dark purple and light purple layers of the banner are obvious (Fig 3a,b). The above results showed that different colored eighth-month beans produced plants with no significant differences in flowering time. However, brown seeds produced plants with light purple flowers, seeds with variegated markings produced plants with dark purple flowers, and black seeds produced plants with white flowers.

3.3 Comparison of pod traits of eighth-month beans

The flowering period of eighth-month beans lasts 2 to 3 days, hence we observed whether there are any differences in the time from withering to pod formation of plants grown from the three different seeds. Table 1 shows that the time from flower withering to pod formation of the three different seeds is 2–2.3 days and there were no significant differences in the time from withering to pod formation among these three different seeds. However, the color of the pods formed showed significant differences (Fig 4). Plants grown from brown seeds produced pods with six different colors: dark purple, light purple, purplish green, white, light green, and dark green (Fig 4a). Plants grown from seeds with variegated markings produced pods that are light green or light purple in color (Fig 4b). Plants grown from black seeds produced only light green pods (Fig 4c). Plants grown from brown seeds produced pods with pod length of 21–29 cm (Table 1), of which light green and dark green pods are the longest while white pods are the shortest. Dark green pods produced the heaviest single pod at 17.5 g and white pods are the lightest at 6.8 g (Table 1). Plants grown from seeds with variegated markings produced pods with pod length of 25–30 cm (Table 1). Light green pods produced the heaviest single pod at 16.2 g, followed by light purple pods at 10.5 g (Table 1). Plants grown from black seeds produced pods with pod length of 31 cm and single pod weight of 15 g. After understanding the differences in pod color and single pod weight, the number of seeds in single pods of plants grown from the three different seeds was compared. Plants grown from brown seeds

produced six different colored pods and the average number of seeds per pod was 15–16. Plants grown from seeds with variegated markings produced two different colored pods and the average number of seeds per pod was 17. Plants grown from black seeds produced an average number of 18 seeds per pod. The above results showed that in terms of pod color, plants grown from brown seeds produced the most variety, but their pod lengths also showed the greatest variation. In terms of pod weight and number of seeds, black seeds produced pods with the greatest number of seeds (Table 1).

3.4 Comparison of anthocyanin content in leaves and pods of eighth-month beans

The anthocyanin content of leaves and pods from plants grown from different seeds were compared to see if there are any differences. Table 2 shows that the anthocyanin content in leaves from plants grown from the three different seeds was undetectable. However, among various colored pods, anthocyanins could be detected in purple pods. Among these pods, the dark purple pods produced from brown seeds showed the highest anthocyanin content at 0.073 ug/mL. In addition, the anthocyanin content of white pods was 0.039 ug/mL. The above results showed that anthocyanins could not be detected from green leaves and pods, and could only be detected in purple or white pods (Table 2).

3.5 Comparison of next-generation seed traits and germination rate of eighth-month beans

The seeds in pods from plants grown from the three different colors of eighth-month beans showed differences in color, shape, and length. Plants grown from brown seeds that produced six different colored pods produced seeds ranging from dark to light brown. On pod maturation and dark purple pods produced the smallest seeds, followed by dark green pods (Fig 5a). Plants grown from seeds with variegated markings produced next-generation seeds with the greatest variation: light-green pods produced dark brown seeds with the smallest shape and length, while light purple pods produced light brown seeds with white markings (Fig 5b). Plants grown from black seeds produced next-generation seeds with the smallest variation, with even shape and length and all seeds were black (Fig 5c). The germination rates of next-generation seeds showed no significant difference, and germination rate was around 45%–55%.

4. Discussion

Anthocyanins are widely present in plants, are flavonoids, and a natural anti-oxidant. Studies have shown that daily consumption of apples with high anthocyanin could effectively prevent disease occurrence (Bagchi and Bagchi, 2007). Butterfly pea (*Clitoria ternatea*) is a leguminous plant that is rich in anthocyanins, and has nephroprotective and anti-carcinogenic activity（Lijon *et al*., 2017; Ramaswamy *et al*., 2011）. Thus, anthocyanins not only could boost anti-oxidative capacity in the human body, but can also prevent diseases. In this study, light purple and dark purple (Fig 4a,b) eighth-month bean pods had an anthocyanin content of 0.028 μg/mg–0.073 μg/mg (Table 2), showing that as purple coloration becomes more intense in pods, anthocyanin content also increases, and hence it could be postulated that anti-oxidative capacity also increases. Anthocyanin content in plants can be induced by the environment. When grape leaves are treated with 0.12 M sucrose or 20 μM abscisic acid, the anthocyanin content is significantly increased, but treatment with 30 mM nitrate could inhibit sucrose-induced anthocyanin（Pirie and Mullins, 1976）. Abiotic stress can also induce production of anthocyanin in plants to reduce the damage of free radicals to plants（Kovinich *et al*., 2015）. Daisies would increase anthocyanin content under conditions of salt stress, in order to remove free radicals and increase salt tolerance（Khavari-nejad *et al*., 2008）. Brown seeds grown in this study produced pods with higher anthocyanin content (Table 2), suggesting that plants grown from brown seeds have higher stress tolerance.

Legume starch can provide 20%–30% proteins that are rich in L-lysine and limiting sulfur-containing amino acids for human body needs（Duranti, 2006; Phillips, 1993）. Hence, in Africa, Latin America, and Asia, legume starch have become a source for protein supplementation（Aykroyd *et al*.,1982）. Legume starch also contains high dietary fibers and resistant starch. These substances not easily digested in the body and after reaction with bacteria inside the intestines to become short-chain fatty acids such as acetates, propionates, and butyrates（Mallillin *et al*., 2008; Costa *et al*., 2006）. Among these fatty acids, butyrates can promote the growth of probiotics in the intestine and lower the risk of colon cancer (Scharlau *et al*., 2009）. Aside from providing essential nutrients to humans, legume starch can also lower cholesterol. Studies have shown that after rats were fedlupine-extracted proteins for two weeks, blood cholesterol was decreased（Sirtori *et al*., 2004）. When patients

with hypercholesterolemia were given 250 g of proteins extracted from cowpeas every day, it was found that after 6 weeks, the total cholesterol in patients decreased by 12%, low-density lipoprotein-cholesterol (LDL-C) decreased by 18.9%, and high-density lipoprotein-cholesterol (HDL-C) increased by 2.7%, suggesting that proteins extracted from cowpeas can effectively decrease formation of LDL-C（Frota *et al*., 2015）. The eighth-month bean in this study belongs to the genus of cowpea, and cowpeas can not only provide proteins and starch as nutrients, but also has health effects（Phillips *et al*., 2003）. Therefore, the nutrient content of eighth-month beans should be valued and further analysis of its health effects should be carried out.

This study uses eighth-month beans as study materials and recorded in detail the agronomic traits and production in various plants grown based on seed appearance. The complete growth data of these plants is sorted and could be used as a reference for future breeding of eighth-month beans.

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Fig. 1. Comparison of seeds traits in eighth-month beans. After the seed harvest, the seed color can be divided (a) brown, (b) variegated markings and (c) black. The lower right scale is 1cm

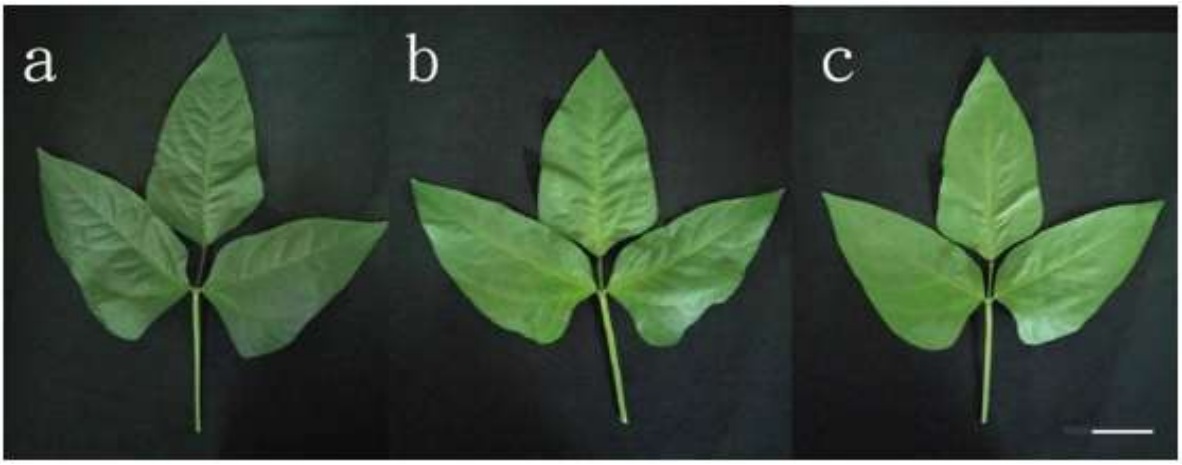

Fig. 2. Comparison of leaf traits in eighth-month beans. After two weeks of eighth-month beans. cultivation, the upper leaves were compared, where (a) the leave were obtained from cultivation of brown seeds, (b) the leave were obtained from cultivation of variegated markings seeds, (c) the leave were obtained from cultivation of black seeds. The lower right scale is 3 cm.

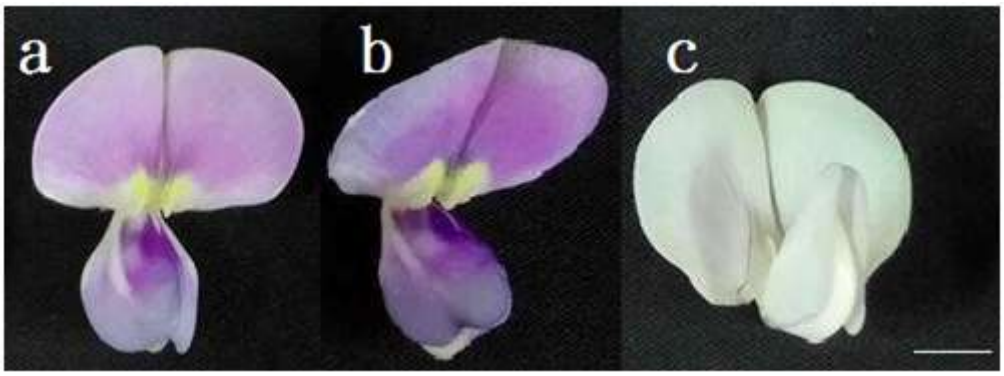

Fig. 3. Comparison of flower traits in eighth-month beans. After eighth- month beans flowering, the upper flower were compared, where(a) the flower were obtained from cultivation of brown seeds, (b) the flower were obtained from cultivation of variegated markings seeds, (c) the flower were obtained from cultivation of black seeds. The lower right scale is 1 cm.

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Fig.4. Comparison of pods traits in eighth-month beans. After three weeks of eighth-month beans. flowering, Take the mature pods for comparison, where (a) the pods were obtained from cultivation of brown seeds, (b) the pods were obtained from cultivation of variegated markings seeds, (c) the pods were obtained from cultivation of black seeds. The lower right scale is 3 cm.

	The offspring of brown seeds						The offspring of variegated markings seeds		The offspring of black seeds
	a						b		c
POD COLOR	Dark purple	Light purple	Purple green	white	Light green	Dark green	Dark green	Light purple	Yellow green
Progeny Seed Germination Rate	45±5 a	45±5 a	50±0 a	55±5 a	40±5 a	45±5 a	55±5 a	50±0 a	45±5 a

Fig.5. Comparison of offspring seed traits and germination rate in eighth- month beans. After ripe pods of eighth- month beans, Take the mature seeds for comparison, where (a) the seeds were obtained from cultivation of brown seeds, (b) the seeds were obtained from cultivation of variegated markings seeds, (c) the seeds were obtained from cultivation of black seeds. Every value expressed by mean ± standard error. Means in the same column followed by the different letter are significantly different ($p<0.05$) by Least Significance Difference. The lower right scale is 1 cm.

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Table 1. Comparison of flowering time, pod time, fruit pod color, fruit pod length, fruit pod weight and seed germination rate of progenies from different eighth-month beans seed after planting.

Seed color	**Brown**						**Variegated markings**		**Black**
Flowering time (days)	39.5±1.9a						39.6±2.3a		40.4±1.6a
Out pod time (day)	2.0±1.2a						2.0±0.0a		2.3±1.7a
Pod color	**Dark purple**	**Light purple**	**Purple green**	**white**	**Light green**	**Dark green**	**Dark green**	**Light purple**	**Yellow green**
Pod length (cm)	22.6±2.9a	26.0±2.0ab	25.5±1.8ab	22.6±2.9a	26.0±2.0ab	25.5±1.8ab	22.6±2.9a	26.0±2.0ab	25.5±1.8ab
Pod weight (g)	9.9±2.3ab	10.6±2.3ab	11.1±3.5abc	9.9±2.3ab	10.6±2.3ab	11.1±3.5abc	9.9±2.3ab	10.6±2.3ab	11.1±3.5abc
Average grain number	16.3±1.9a	16.0±1.6a	15.2±2.5a	16.3±1.9a	16.0±1.6a	15.2±2.5a	16.3±1.9a	16.0±1.6a	15.2±2.5a

(1) Every value Expressed by mean ± Standard Error.

(2) Means in the same column followed by the different letter are significantly different (p<0.05) by Least Significance Difference.

Table 2. Comparison of the content of anthocyanin in pods with different eighth- month beans. seeds after planting.

Material / Seed color	Leaf		Pod	
	Color	The content of anthocyanin (μg/mL)	Color	The content of anthocyanin (μg/mL)
Brown	Dark green	N.D.	Dark purple	0.073±0.017 a
			Light purple	0.028±0.008 b
			Purple green	0.033±0.005 b
			white	0.039±0.009 ab
			Light green	N.D.
			Dark green	N.D.
Variegated markings	Light green	N.D.	Dark green	N.D.
			Light purple	0.026±0.007 b
Black	Light green	N.D.	Yellow green	N.D.

(1)N.D: not detected
(2)Every value Expressed by mean ± Standard Error.
(3)Means in the same column followed by the different letter are significantly different (p<0.05) by Least Significance Difference.