GERMINATION, PROPAGATION AND SELECTION OF *ANDROGRAPHIS PANICULATA* FOR FUTURE BREEDING PROGRAMME

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ABSTRACT

Andrographis paniculata (hempedu bumi) is one of the most popular medicinal plants used traditionally for treatment of various diseases such as diabetes, cancer and malaria. This herb that familiar as king of Bitters was reported possessed a various compound especially in its aerial parts and roots as both have been widely used as traditional treatment. Naturally, this herb grows well in most soil types and it is widely distributed in different regions of Southeast Asia, China, America, West India and Christmas Island. As this herb getting attention for pharmacology activities and also listed as one of the herbs under National Key Economic Area (NKEA), it has to be protected and placed as genetic resources (germplasm). For these reasons, Forest Research Institute Malaysia (FRIM) have took initiative by doing a collection of A. paniculata from five selected populations such as Johor, Pahang, Perak, Perlis and Terengganu. The collection has covered Peninsular Malaysia and representative region of north, east, west and south. All the accessions were hardened at nursery before they were propagated via stem cuttings and seeds. The plants were then multiplied to get uniform planting materials before it can be planted in a germplasm plot. Results from stem cuttings showed that 80% of cuttings were survived. Sources from seeds also were tested at nursery and laboratory conditions in order to evaluate germination rate. However, results showed that germination under laboratory conditions revealed higher percentage (61%) compared to nursery (38%). Prior to germplasm establishment, clones propagated via stem cuttings were choosen as their survival rate is higher than seeds. All propagated plants were tested at different growing media with addition of organic matter (such as sawdust, cocopeat, compAcc) and tested at different growing conditions (open and shade) in order to identify the best growing media for the plant growth. The propagated plants from the five populations were then been planted in a germplasm to ensure sustainability of A. paniculata. After six months of planting, High Performance Liquid Chromatography (HPLC) analysis was carried out to quantify the reference marker (andrographolide) from the five populations. Results indicated that concentration of andrographolide are higher (more than 9.0 µg/ml) in three populations, Terengganu, Pahang and Perak. These populations have high potential to be selected as sources of production of high qualities planting materials in the future. In a conclusion, germination, propagation and selection is important for species development so that the it can be used for future breeding activities. In future, it was hoped that cultivars with good phenotypic and yield could be determined through chemical markers identification and biomass evaluation.

Keywords: Cuttings, seeds, germplasm, performance, hempedu bumi

INTRODUCTION

The global demands for the herbal products have increased due to unique benfits of the herbs that has been practiced in the past and being used recently as health supplmements, cosmetics and beauty products. This phenomenon has increase awareness among Malaysians regarding the importants of herbs. The Malaysia market for herbs products also has increase at a rate of 15-20% per anmum and is projected to reach US $ 9.4 by 2020 (Rohana et al, 2017). Nowadays, Malaysia government has seriously bring forward the herbal sector into global market by strengthen the activities of herbal products starting from upstream to downstream. Eighten high value herbal products has been listed under Entry Point Project (EPP1) for this purpose and one of the herbs, *Andrographis paniculata* or Hempedu bumi was selected as main target for this study.

Andrographis paniculata Andrographis paniculata is a softwood herbaceous plant from family Acanthaceae, It is an important medicinal plants used since ancient for treat various health ailments such as snake bite, bug bite, diabetes, dysentery, fever and malaria (Md. Sanower Hossain et al. 2014). The different parts of *A. paniculata* have their own medicinal uses. In Malaysia, aerial parts were mostly used and the water decoction effectively to treat common cold, hypertension, diabetes, cancer, malaria and snakebite (Okhuarobo, 2014). This plants also known as King of Bitters due to its extremely bitter taste.

For its morphology, *A. paniculata* has stem of about 30-90 cm high, erect, smooth thickened at lowed nodes, smooth with numerous long divaricats branches. The leaves are opposite and produce numerous small flowers. The fruits are 0.5 to 1 inch long and acute at both end whereas seeds are numerous (6-10 per capsule), glabrous with yellowish brown colour. This herb is native to peninsular India and Srilanka and distributed in different regions of Southeast Asia, China, America, West Indies and Christmas Island. It easy to grow and sometimes they considered it as weed. Their habitat commonly found in wasteland, grassland, backyard gardens or pots. It is very hardy plant, survive very well, and adapt itself in a variety of soil conditions (Forestry Department Peninsular Malaysia).

Nowadays, many plant species have been revalued their therapeutic chemical including this herb *A. paniculata* as the effect of numerous problems associated with orthodox drugs. For that, many phytochemical studies have found that this herb contains diverse compounds such as labdane diterpenoid lactones, flavonoids and miscellaneous compounds. All these possess contribution to pharmacological properties such as anti-microbial, antioxidant, anti-infalmmatory, anti-diabetic and insecticidal activities (Mishra, Sangwan and Sangwan, 2007). A major bioactive compound in *A. paniculata* is Andrographolide. This compound has shown anticancer potential in various research. Recently, this herb getting attention and demand for pharmaceutical due to these discoveries.

However, there are many problems in getting large quantity of raw materials with high quality. Mostly this herb was collected from wild and surround residential area. Originally, it is propagated using seed but they might have different appearance in different locations and low germination rate. The information available on seed quality and germination very meagre. Therefore, Forest Research Institute Malaysia (FRIM), has tried several experiments to grow the best *A. paniculata* by seeds and others propagation cutting method in order to get the standardization for growing this species vigorously and establishing *A. paniculata* germplasm that can be used a reference centre and for breeding activities. This study were conducted with the objectives of i) to determine the germination rate of seeds *Andrographis paniculata* under laboratory condition and nursery condition, ii) to determine the viability of *Andrographis paniculata* using stem cuttings, iii) to determine the suitability of growing medium for *Andrographis paniculata* and iv) to identify the suitability of growing *Andrographis paniculata* under open and shade. It is expected, output from the study will help in provide information and could be used by others especially herbal planters for establish *A. paniculata* germplasm and grow it well in certain condition to meet the requirement from herbal industry.

MATERIALS AND METHODS

Collection *of Andrographis paniculata* From Different Populations

A total of 10 mother plants of *Andrographis paniculata* were collected from different five populations in Peninsular Malaysia. All mother plants were wrapped and transported back to FRIM. The five populations were coded as JHB (from Kota Tinggi, Johor), CHB (from Kuantan, Pahang), AHB (from Tapah, Perak), RHB (from Mata Ayer, Perlis) and THB (from Kuala Terengganu, Terengganu). Each collected location were tagged using Global Positioning System (GPS). The topographic information such as location coordinates and altitudes were also recorded (Table 1). The morphological characteristics of all mother plants such as height, diameter, leaf length, and leaf width were measured and recorded as shown in Table 2.

Table 1: Topographic information of *Andrographis paniculata* from five populations

Populations	Code	GPS Points	Altitude
Kota Tinggi, Johor	JHB	N2 18.329 E102 37.015	20 m
Kuantan, Pahang	CHB	N3 46.281 E103 14.061	8 m
Tapah, Perak	AHB	N4 12.369 E101 16.085	59 m
Mata Ayer, Perlis	RHB	N6 26.450 E100 16.470	19 m
Kuala Terengganu, Terengganu	THB	N5 25.915 E103 03.071	16 m

Table 2: Morphological characteristics of *Andrographis paniculata* mother plants from five populations

Population	No. of clumps	Height (cm)	Diameter (mm)	Leaf length (cm)	Leaf width (cm)
JHB	3-14	22-102	1.8-11.9	4.5-11.5	1.0-3.7
CHB	2-14	29-120	2.3-10.7	5.0-13.0	1.0-3.6
AHB	3-15	36-89	2.3-11.2	3.6-14.5	1.0-3.8
RHB	2-15	24-87	2.3-8.7	5.0-13.0	1.1-3.6
THB	1-10	23-90	2.4-7.0	3.0-10.3	1.0-4.9

Maintaining of *Andrographis paniculata* at Nursery Stage

After mother plants were bring back to FRIM's Nursery, it is vital to maintain these plants at nursery condition so that they get the correct initial care and acclimatized for their long lives. It is also the process to understand their needs such as fertilization requirement and water supply. All mother plants were planted in a yellow tray (9.5 cm height x 40 cm width x 53 cm length) and maintained for one month before multiplication of these plants by propagation method was done.

Germination of *Andrographis paniculata* Seeds

Seeds from five populations of *A. paniculata* were collected from mother plants that were raised at nursery. Matured seeds collected were germinated at two different conditions, which is nursery and laboratory. Under nursery condition, seeds of *A. paniculata* were taken out from its capsule/ pod and temporary placed in petri dish. 100 viable seeds per accessions were germinated in a germination tray consisted of river sand. A slight layer of sand was applied to fully immerse them in sand. Observation was done two days of time interval for at least 30 days.
Under laboratory condition, seeds of *A. paniculata* were taken out from its capsule/ pod and temporary placed in petri dish. Then, 100 viable seeds were selected from each accession. Infected and infested seeds were removed, only clean seeds were selected randomly for further experiments. A germination test was performed with four replicates of 100 seeds each, distributed on paper towels, moistened with water and placed in germination room at 28°C. The germination process was done under light (using white fluorescent lamps). Assessment was performed according to the rules for seed analysis by International Seed Testing Association (ISTA). The seedlings that presented open cotyledon leaves were counted and the results were expressed in percentage. Observation was done two days of time interval for at least 30 days.
Germination rate under both conditions for every week and per populations were plotted in a graph.

Propagation of *Andrographis paniculata* By Stem Cuttings

Besides seeds, herbal such as *A. paniculata* also can be propagated using stem cuttings. Only matured stems from mother plants were selected as propagation material. The stems were cut slanted within 5 cm to 7 cm and dipped into rooting hormone to stimulate the root growth. The stems were planted into the planting bed that contains 100% sand media. Successful of rooting by stem cuttings, number of roots and root length were recorded and calculated. Data was analysed using one-way ANOVA, Minitab version11.

Growing Stem Cuttings in Different Media and Different Growing Conditions
The rooted stem cuttings were transferred to the polybags size of 8" x 8" and tested at different growing media containing four treatments. The four treatments used are:

i. **Treatment 1 :** 2 topsoil : 1 sand (control)

ii. **Treatment 2 :** 2 topsoil : 1 sand : 1 sawdust

iii. **Treatment 3 :** 2 topsoil : 1 sand : 1 cocopeat

iv. **Treatment 4 :** 2 topsoil : 1 sand :1 CompAcc

Then, the polybags were placed at different growing conditions with 50% shade and open area. After a month, morphological data such as leaf number, leaf width, leaf length and collar diameter were collected and recorded. Data were analysed using one-way ANOVA, Minitab version 11.

Establishment of Germplasm
The plants that survived and grow well were transferred to a big pot. Each pot contained five plants of *A. paniculata* resulted from stem cuttings of the same population. Each population have 10 replications. They were placed under open area and manually monitored. This germplasm will be used for future breeding programme and any research as no much research related to this species has been done or published. However, quantitative analysis on chemical constituent of *andrographolide* have been carried out for the first phase after establishment (after 6 months) in order to select high yielding populations.

Figure 1: Layout design of *Andrographis paniculata* germplasm

JHB	JHB	CHB	CHB	AHB	AHB	RHB	RHB	THB	THB
JHB	JHB	CHB	CHB	AHB	AHB	RHB	RHB	THB	THB
JHB	JHB	CHB	CHB	AHB	AHB	RHB	RHB	THB	THB
JHB	JHB	CHB	CHB	AHB	AHB	RHB	RHB	THB	THB
JHB	JHB	CHB	CHB	AHB	AHB	RHB	RHB	THB	THB

Quantitiave Analysis of Andrographolide

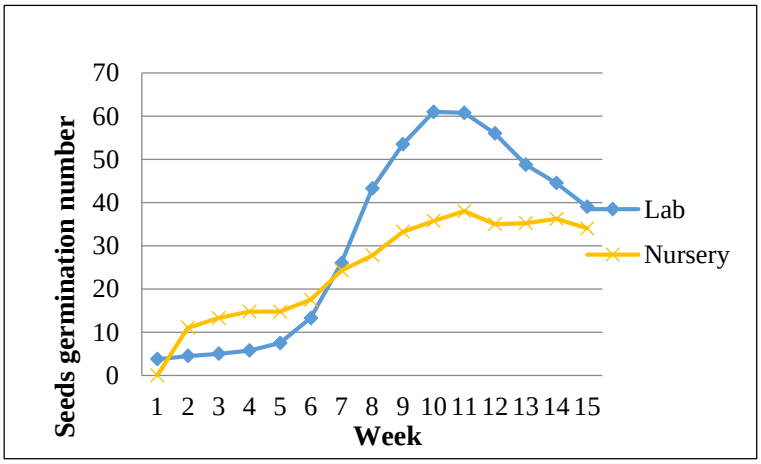
A total of 0.5 g of sieved powder material (500 µm) from five populations. About 5 mL of methanol was added and the mixture was ultra-sonicated for 15 minutes. The resulting solution was filtered using 0.45 µm syringe filter prior to analysis. The samples were analysed by means of a HPLC system (Waters 2535 quarternary gradient module, Waters 2707 Autosampler and Waters 2998 photodiode array detector). A Phenomenex Luna C18 column was used (4.6 mm i.d. x 250 mm) and for elution of the constituents, three solvents denoted as A, B and C were employed. A was 0.1% aqueous formic acid, B was acetonitrile and C was methanol. The flow rate used was 1.0 mL/min and the injection volume were 10 µL. The retention times and UV spectra of the targeted compounds were analysed at wavelength of 220 nm.

RESULTS AND DISCUSSIONS

Germination of *Andrographis paniculata* Seeds

Normally, germination of seeds was started within 3 days on first week. In this study, seed germination of *A. paniculata* were counted once in two days interval for both nursery and laboratory conditions. Germination graph per weeks was represented in Figure 2. Based on observation, all seeds germinated in laboratory sprout faster than in nursery condition after four days. However, number of seeds sprout in nursery condition were increased started from week 2 until week 7 compared to the seeds in laboratory condition. Then, seeds sprout in laboratory started to increase again on week 7 until 10 and after that their survival started to decreased. Compared with seeds in nursery, they were germinated slowly until reached week 15 but the survival is low.

Figure 2: Number of seed germination of *Andrographis paniculata* by weeks



From this study, it can be said that seed germination of *A. paniculata* very in low rate because this plant normally reaches a maturation stage up to 80 to 100 days of germination (Daryush et al., 2012). Other factors may also contribute to it. The availability of water, seeds vigour, time, temperature and heat, presence of oxygen and exposures to light play an important role in the germination process.

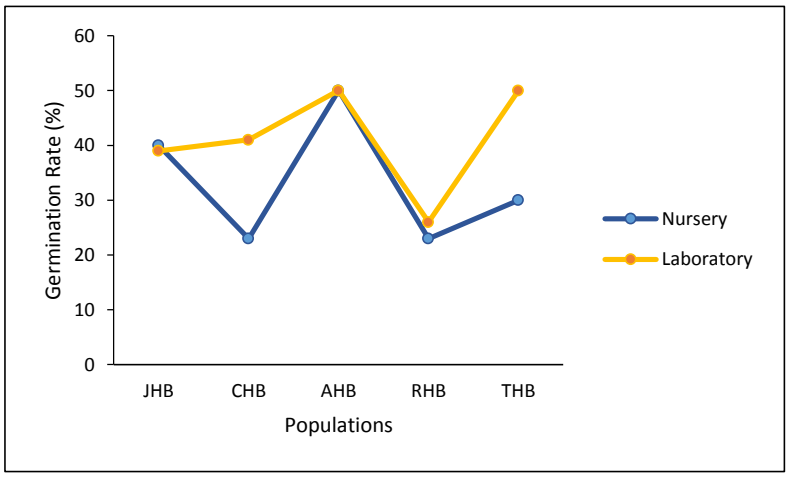
Condition prepared such as in laboratory was a controllable condition where is the best condition to supply enough and consistent sources for seed to germinate. While under nursery condition, it cannot be control because it is under natural condition where the weather and environmental condition may be not consistent from days to days. It may influence the seed germination of *A. paniculata* since the seeds is very small size. According to Mojtaba et al. (2016), large seeds normally had higher germination rate, seedling emergence success and more rapid growth than small seeds because large seeds has higher protein synthetizing ability for protein synthesis.

Besides that, the shape and the size of the trays and Petri dish may be not suitable enough for 100 seeds shared together within a long time period which about 4 months duration. The limitation of the space also plays an important role in germination process.

This is because they need to compete each other to get sources to survive longer. That is why the seed germination decreased when they reach at maturation stage.

Meanwhile in Figure 2 shows the germination rate of *A. paniculata* seeds from different populations under nursery and laboratory conditions. Graph showed germination rate of AHB seeds from Tapah, Perak recorded higher (50 %) for both conditions. Whereas, JHB seeds from population of Kota Tinggi, Johor revealed about 39% for laboratory condition and 40% for nursery condition. For seeds THB and CHB from populations of Kuala Terengganu, Terengganu and Kuantan, Pahang, germination rate were higher in laboratory condition compared to nursery. However, low rate of seed germination has been revealed by RHB seeds from population of Mata Ayer, Perlis.

Figure 2: Number of seed germination of *Andrographis paniculata* by populations



Propagation of *Andrographis paniculata* By Stem Cuttings

Due to low of germination rate from seeds of Terengganu and Perlis. Alternative has done by propagated *A. paniculata* by stem cuttings for both populations. Results in Table 3 showed propagation through stem cuttings is more successful. Survival rate for both more than 80%. No of roots formed is within 5.0 cm with the length between 2.0 cm. This study shows that stem cuttings *A. paniculata* of is more preferable, easy to get, and convenient method in order to obtain high quantity of planting materials.

Table 3: Survival, mean number of roots and root length of *A. paniculata* stem cuttings

Populations	Parameters		
	Survival (%)	No. of root	Root length (cm)
Perlis	87	4.93a ± 0.78	2.16a ± 0.19
Terengganu	80	4.86a ± 0.41	1.79a ± 0.17

Means followed by same letter is not significant at 0.05 level of significance.

Growing Stem Cuttings in Different Media and Different Growing Conditions

The survival and successful stem cuttings from Perlis were selected for this study to determine the suitability of growing medium for *A. paniculata* and thus to identify the suitability of growing this species under open or shade area. A total of 30 stem cuttings were used for per treatment of growing media. Results of *A. paniculata* in different media under open and shade were presented in Table 4.

Table 4: Mean of *A. paniculata* growth parameters in different treatmwnt of growing media under open and shade area.

	Treatment	No. of leaf	Leaf length (cm)	Leaf width (cm)	Collar diameter (mm)
Open	Control	3.50b±1.00	3.14a±0.47	0.78ab±0.10	2.05a±0.15
	Sawdust	-	-	-	-
	Cocopeat	4.25b±0.75	3.75a±0.58	0.98a±0.13	2.43a±0.11
	CompAcc	14.53a±1.73	3.05a±0.19	0.61b±0.05	1.33b±0.11
Shade	Control	11.19a±2.12	3.86a±0.31	0.95a±0.08	1.89a±0.14
	Sawdust	-	-	-	-
	Cocopeat	4.43b±1.80	3.07a±0.65	0.64a±0.15	1.42a±0.18
	Compacc	11.47a±1.34	3.45a±0.27	0.85a±0.09	1.90a±0.17

Means followed by same letter is not significant at 0.05 level of significance.

Based on the results from analysis of variance (ANOVA) shown in Table 4, all stem cuttings in sawdust treatment that were placed under open and shade were died. It means this treatment of growing media were not suitable for this species as it take moderate drainage.

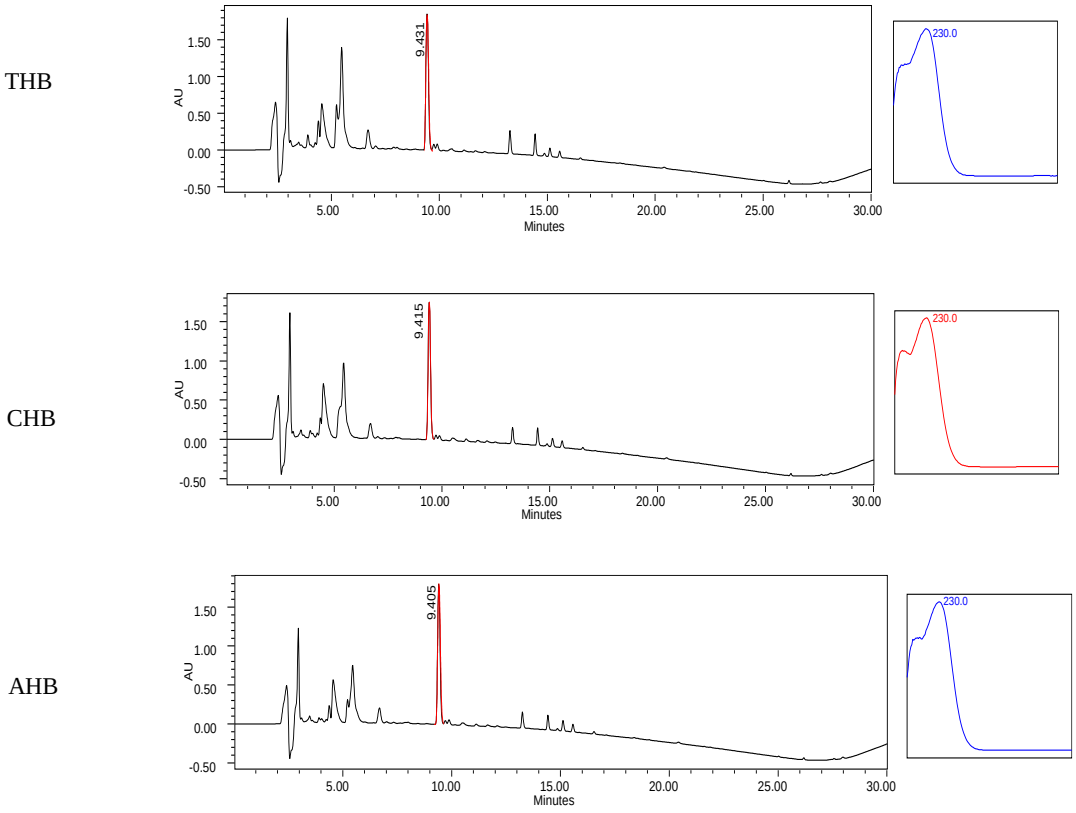
For other parameter such as number of leaves, it shows that treatment of CompAcc media recorded the highest (14.53 cm ± 1.73) under open area at three months. This might be due to as stated by Do and Scherer (2013), composts (compAcc) originating from different organic wastes were considered as a beneficial and excellent partial substitution for peat in term of its high abundance and as an improver of soil characteristics and plant growth in horticulture. Producing a compost as organic fertilizer with higher nutrient content would be quite promising not only in providing greater stability in production, but also in maintaining better soil fertility. Similar finding with the stem cuttings placed under shade. However, number of leaves for stem cuttings with compAcc treatment (11.47 cm ±1.34) under shade did not significant with the control (11.19 cm ±2.12). In treatments of cocopeat for open and shade, stem cuttings produce lower number of leaves.

In term of leaf length, stem cuttings did not show any significant value in all treatments of growing media either under open and shade. Similar with leaf width under shade. Stem cuttings did not show any significant value in all treatments used. While for open, stem cuttings produce maximum leaf width and collar diameter from cocopeat growing media. Thus, this study has found that CompAcc is the best growing media for the herbal such as *A. paniculata.*

Chemical Constituents of Andographolide

All samples from five populations exhibit the andrographolide constituent. Samples from Terengganu (THB), Perak (AHB) and Pahang (CHB) contained higher concentration of andrographolide as compared with other populations (Figure 3). The concentration of andrographolide showed more than 9.0 µg/ml and present during injection time nine to ten minutes. The concentration obtained in this study is higher if compared to the results obtained by Ibrahim & Chong (2008) reported that the concentration of andrographolide is more than 7.0 µg/ml under different dried sample storage temperature. However, the chemical consituents from the five populations in this study will be further analysed until the age of 12 months in order to identify the optimum production of andrographolide for this species.

Figure 3: HPLC Chromatogram of andrographolide in *Andrographis paniculata* from 6 months planting materials



CONCLUSION

As a conclusion, it was observed that seeds of *A. paniculata* have low germination rate. Improvement in seed germination of this species has been done and to take seriously so that development of standardization or protocol for growing this species could be used by others so that it can be expand into the large field. Other than that, stem cuttings also can be used as an alternative in produce the high quantities of plating materials. This method suitable for clone the superior of *A. paniculata* genotype with

desirable characteristics. In order to establish germplasm for future breeding activities, the needs and requirements of growing this species very well have to be calculated so that the sources of quality planting materials can be sustained. All three populations that presented high concentrations of adrographolide will be nurtured well for multiplication because they have high potential to be selected as high quality planting materials in the future.

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