Intraspecific Crosses and Morphological Studies of two cultivars of *Vigna radiata* through *In Vitro* and *In Vivo* Techniques

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Abstract: *Vigna radiata* or Mung bean (known as Green gram) and *Vigna mungo* or Black eye pea (known as Black gram) are highly valued plants for human and animal food. The genus *Vigna* is a pan-tropical comprising about 170 species. This study is being conducted using *in vitro* and *in vivo* techniques for evaluating morphological characters and also the characteristics of the interspecific hybridization between these two species. The study showed that there was no significant difference in terms of plant height, the number of pods per plant and the number of seeds per pod for these two species in *in vitro* and *in vivo* conditions. In *in vivo* method, there were also no significant differences in branch length, growth vigor, days to first flowering, plant height at flowering stage, days to first ripening, days to 50% mature pod, nodes per plant, fertile nodes per plant, percentage of fertsile nodes, pods per fertile nodes, pod length, seed length and the size of leaf except they differ in days to emergence, flowers per node and the seed coat color. In *in vitro* method, the same results were obtained. Furthermore, *in vivo* technique gave the highest yield and yield components compared to *in vitro* technique. Crosses between these two species showed that the most compatible cross in relation to the percentage of pods set and normal mature seeds per pollination were *Vigna radiata* x *Vigna mungo* when *Vigna radiata* was used as female parents. Moreover, the yield was increased and shiny green seed coat was produced through this technique.
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1. Introduction

Legumes are vastly grown in the world and their economic importance is distinguished worldwide. They are economical source of proteins for humans that add variety to the diet (Kakati et al., 2010). The genus *Vigna* Fabaceace is composed of 200 species and *Vigna* is encompassed of 20 species that are native to warm and tropical regions of the world. *Vigna* species serve as pot herbs, vegetables, hay, pasture, green manure, soil cover and improvers. Green gram (*Vigna radiata*) and black gram (*Vigna mungo*) are two of the most imperative food legumes grown and consumed (Kakati et al., 2010).

For growing Mung bean, the seedbed should be pulverized so that the seed comes into close contact with the soil which has moisture available for rapid seed germination. Mung bean germinates rapidly and emerges within three to four days after sowing with favorable temperature and soil moisture.

An initial step in genetic engineering with a particular crop species is the development of techniques for *in vitro* regeneration of functional plants from tissue fragments, embryonic tissue, calli, protoplasts or isolated cells. Generally, it has been more difficult to regenerate functional plants from grain legume species. Only minor attention has been devoted to tissue culture research in the Mung bean. Plant tissue culture is a technique in which cell, tissue or organ of a certain plant can be cultured in the nutrient media that is prepared in an aseptic condition without the presence of microorganisms in a controlled manner (Seabrook, 1980). Nutrients obviously are important factors in cell and tissue culture (Bonga and Aderkas, 1992). The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used (George and Sherrington, 1984). Components of the media for the growth of plant can be classified into six groups including major inorganic nutrients, trace elements, iron source, organic supplement (vitamins), carbon source, and organic supplement (plant growth regulators) (Dixon, 1985).

The Mung bean seed coat has two layers containing an outer columnar layer that may contain deep blue or black pigment and a green parenchyma layer which contain various concentrations of chloroplasts (Watt, 1975). Watt (1975) found that the dull-rough seed surfaces are because of a seed texture

layer originating from the inner pod membrane that covered glossy-smooth seed coats.

Mathews et al. (1986) regenerated functional plants from embryonated cotyledons of Mung bean. The cotyledon explants were cultured on MS basal medium without addition of exogenous hormones. Plants with shoots and roots arose from the proximal end of the cotyledon within 25 to 30 days. After transplanting into sterile soil the plants matured and produced viable seeds. When the seeds were planted, 14% of the progenies exhibited a wide spectrum of chlorophyll or morphological mutations similar to those obtained following seed treatment with radiations or chemical mutagens (Mathews et al., 1986). This study was conducted to estimate different morphological characteristics of *Vigna* cultivars through *in vitro* and *in vivo* techniques and intraspecific hybridization traits.

2. Materials and Methods

This experiment was conducted in the greenhouse of the Institute of Biological Sciences, Faculty of Science, University of Malaya.

Two types of *Vigna radiata* cultivars were used including dark green (DG 04) and shiny green (SG 05) and embryos were collected from the inner cotyledons of the seeds.

2.1. Culturing methods

2.1.1. *In vivo* technique

A split plot design with 3 replications was used in this experiment. Two Mung bean varieties, dark green (DG 04) and shiny green (SG 05), were evaluated. They were grown in plots based on their difference with appropriate labeling. Each plot contained 20 black plastic bags with black sandy loam soil and one seed was sown per plastic bag. The plot size was 150 x 60 cm and there were two rows per plot, with spacing of 25 cm between rows and 10 cm between plants. The seedbed for Mung bean was well pulverized so that the seed came into close contact with the soil to have moisture available for rapid seed germination.

Irrigation was applied everyday, once in the morning and once in the evening. Insecticides and fungicides were sprayed 6 times and fertilizers were applied twice along the experimental observation.

Ten plants from each plot were randomly selected for data collection on the following parameters: Plant height (cm), Plant height at flowering stage, Number of days to first flowering, Number of days to first ripening, Number of pods per plant, Number of seeds per pod, Number of days to 50% mature pod and Germination percentage.

2.1.2. *In Vitro* technique

MS (Murashige and Skoog, 1962) media with the combination of two different concentration of hormone BAP (6-benzilaminopurene) were used in the study, MS alone (MS^o), MS + 2 BAP (MS + 2.0 mg/l BAP), MS + 4 BAP (MS + 4.0 mg/l BAP).

The pH of the media was fixed by using 1M HCl (hydrochloric acids) or 1M NaOH (sodium hydroxides). Then, the media was autoclaved within 120^{o}C in 1.2kg/m pressure for 25 minutes. Hot media, still in the liquid phase (50^{o}C) were poured in the sterile culture bottle in 6.5 cm diameter and 8.5 cm height under aseptic condition in the laminar flow chamber.

In the preparation of the basal media (MS), firstly all substances were boiled, then poured in beaker; followed by autoclave at the same temperature and pressure for the preparation of the hormonal media. After that, all the beakers were taken out, poured in several culture bottles and cooled down in room temperature. Embryonic culture was done once the media were already cool and in a solid phase.

In this study, there were 5 embryos cultured per bottle. So, this experiment consumed 150 cultured embryos at once experiment. Cultured embryos in the bottles were kept with optimum light exposure for photosynthesis process, with the room temperature ranges from 25^{o}C to 27^{o}C for tissue development and rapid growth.

2.2. Acclimatization and maturation

When the plantlet reached their height 4 to 5 cm and the root length was about 5 cm, the plantlets were then transferred to the soil. Plantlets were soaked in the 0.5% (w/v) fungal disinfectant solution for 2 to 3 minutes to prevent microbial contaminations and infections.

Plantlets which were grown successfully in the medium were transferred into soil with balance of loam: sand (3:1). The plants were kept in the culture room within room temperature for two weeks for adaptation. A part of them were transferred to the garden for maturation. Watering was done 2 to 3 times daily. Observation was done until maturity was achieved. All data were collected based on the parameters mentioned before.

2.3. Intraspecific hybridization and crossing procedure

Intraspecific hybridization was done by crossing between *Vigna mungo* (black gram) and *Vigna radiata* (Mung bean). Basically, pollen from *Vigna mungo* were crossed with *Vigna radiata* stigma and pollen from *Vigna radiata* were crossed with *Vigna mungo* stigma for this observation.

Emasculation (removing pollen) was performed by pushing one side of the standard and the corresponding wing petal outward with a dissecting needle and removing one-half of the keel petal and the anthers with forceps taking care not to injure the stigma. For the pollen source, mature

flowers were selected in which the anthers dehisced and covered the stigma with fresh ripe pollen grains. The pollen covered stigma was brushed lightly across the stigma of the emasculated flower to complete the pollination. Crossing was done by emasculating in the evening and pollinating the following morning in which the bud was separated completely along the dorsal edge exposing the reproductive organs to fluctuations in the microenvironment. Here, another technique was applied in which the tip of the bud was opened, the anthers were gently removed with forceps and the stigma was pollinated by brushing it with a pollen-laden stigma from the male parent. The opening was sealed with cellophane tape after pollination to prevent drying out of the floral organs.

3. Results

According to the observations, all results were summarized in the graft form in order to show the morphological characteristics comparison between 2 cultivars of *Vigna radiata*, SG05 and DG04, cultured in *in vivo* and *in vitro* conditions as well as the hybridization characteristics between *Vigna radiata* and *Vigna mungo*.

Height of the plants were measured weekly through *in vivo* cultivation method in order to observe differences between the two cultivars.

In the first week, the height of both DG04 and SG05 plants were 12cm. This rate was increased week by week as this parameter was reached to 18cm for both cultivars in the second week. This trend was enhanced steadily and reached the peak of 33cm in the week seven (Fig. 1).

In terms of the number of pods per plant, each of 8 SG05 and DG04 plants showed the lowest number of pods (5). In 10 plants of SG05, 6 pods per plant were emerged while 6 DG04 plants had 6 pods. The highest number of pods (10) was occurred in 5 and 2 plants of SG05 and DG04 respectively (Fig. 2).

The lowest number of seeds per plant (5) was detected in 3 and 1 plant of SG05 and DG04 respectively while 9 seeds per pod were obtained in 10 and 8 plants of SG05 and DG04 which was the highest (Fig. 3).

In terms of the number of days to first flowering, only 1 plant of SG05 had the first flower after 32 days while DG04 did not have any flower yet. After 33 days, 9 plants of SG05 and 5 plants of DG04 had their first flower. The highest number of SG05 and DG04 plants (19) showed their first flower after 35 days. With the passage of time, only a few number of plants flowered firstly (Fig. 4).

As far as the number of days to first ripening concerned, 17 and 14 plants of SG05 and DG04 had their first ripening after 50 days but before or after this time the lower number of plants showed their first ripening (Fig. 5).

In *in vitro* culture, 75% of SG05 plants were germinated while 29% of DG04 plants were germinated and the rest were not germinated or contaminated (Figs. 6 and 7).

Morphological traits of SG05 and DG04 plants which were grown through *in vitro* technique were assessed (Tables 1 and 2). SG05 plants had the average height of 23.7cm which was higher than that of DG04 (22.1cm). In terms of the number of days to first flowering, in average, it took 30 days for SG05 to have first flower while DG04 needed 31 days to flower. In plants flowering stage, the average SG05 height was 22.1cm but this parameter for DG04 was 20.3cm. Both of the cultivars showed the similar average number of pods per plant (3) and the average number of seeds per pod was 6 for both cultivars. In terms of the average number of days to first ripening, it took 41 and 42 days for SG05 and DG04 to have first ripening respectively. SG05 and DG04 showed 50% of mature pod after 36 and 38 days of planting respectively.

Interspecific crosses between *Vigna radiata* (MB) and *Vigna mungo* (BG) were conducted in this study (Table 3). The highest number of pollination was occurred when MB was used as a female and BG was applied as a male (134). In terms of the fallen flowers, MB as a female and BG as a male had a lower number flowers fallen (69) in comparison with MB and BG as a male and female respectively (74). Female MB and male BG showed a higher pod set number which was 65 compared with male MB and female BG (20). The highest pod set percentage (48.5) was obtained when MB and BG were female and male respectively.

According to Table 4, MS media alone and MS with 2BAP gave the highest embryo germination compared with the MS media with 4 BAP. This was probably due to incompatible chemical substance such as high concentration of BAP that inhibited the proliferation of embryo.

Figure 1. Height of SG05 and DG04 plants grown in *in vivo* condition

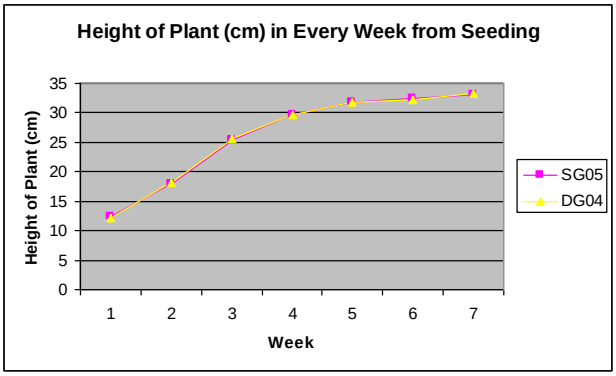

Figure 2. Number of pods per plant in SG05 and DG04 grown through *in vivo* technique

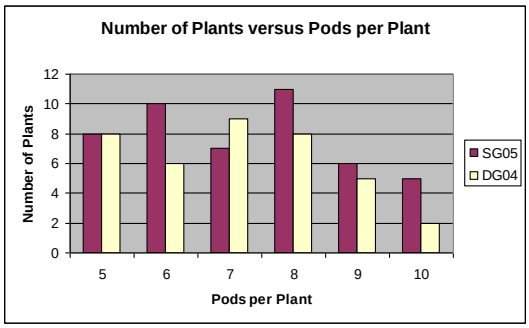

Figure 3. The number of seeds per pod in SG05 and DG04 plants grown in *in vivo*

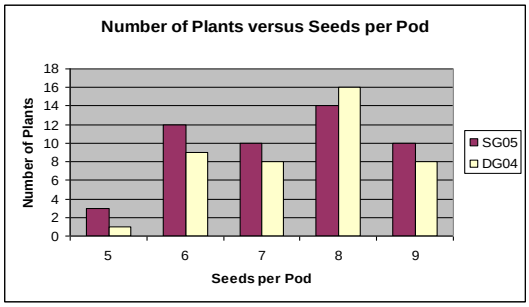

Figure 4. The number of days to first flowering in SG05 and DG04 plants grown in *in vivo*

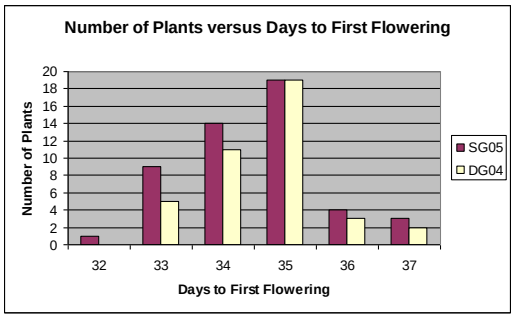

Figure 5. The number of days to first ripening in SG05 and DG04 plants grown in *in vivo*

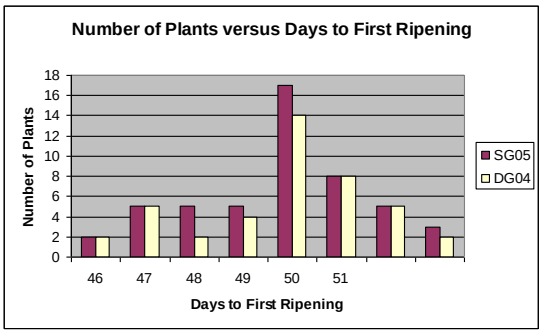

Figure 6. Germination percentage of SG05 through *in vitro* technique

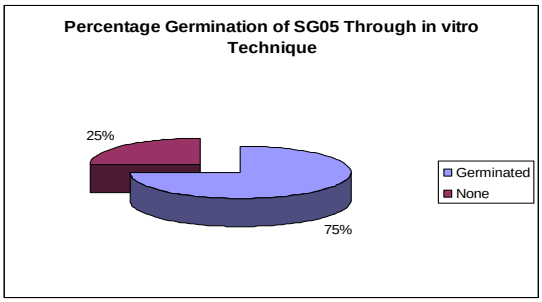

Figure 7. Germination percentage of DG04 through *in vitro* technique

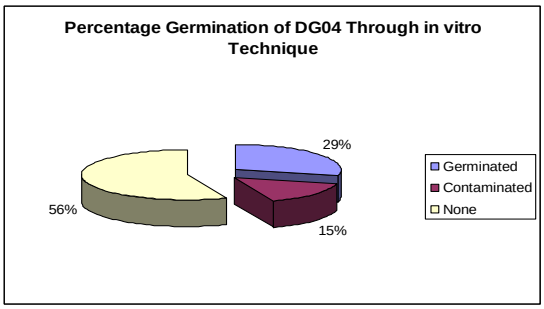

Table 1. Morphological studies of 10 SG05 plants grown through *in vitro* technique

Plants number	Plant height (cm)	Days to first flowering	Plant height at flower (cm)	Pods per plant	Seeds per pod	Days to first ripening	Days to 50% mature pod
1	23.0	30	22	3	6	41	36
2	20.0	30	19	4	6	42	37
3	17.5	28	15	4	5	42	36
4	30.0	32	29	3	8	41	36
5	25.0	33	23	3	5	41	37
6	30.0	34	28	3	8	40	36
7	27.0	33	25	4	7	40	36
8	-	-	-	-	-	-	-
9	26.0	32	24	3	7	41	37
10	15.0	30	14	3	6	41	37
Mean	23.7	30	22.1	3	6	41	36

Table 2. Morphological studies of 10 DG04 plants grown through *in vitro* technique

Plants number	Plant height (cm)	Days to first flowering	Plant height at flowering stage (cm)	Pods per plant	Seeds per pod	Days to first ripening	Days to 50% mature pod
1	19.0	31	18	3	5	43	38
2	23.0	31	21	2	6	43	38
3	17.5	30	15	3	6	42	36
4	-	-	-	-	-	-	-
5	25.0	33	23	3	7	42	39
6	30.0	31	28	3	8	40	38
7	27.0	31	25	4	7	40	37
8	15.0	34	14	4	6	42	36
9	-	-	-	-	-	-	-
10	20.0	31	18	3	6	41	37
Mean	22.1	31	20.3	3	6	42	38

Table 3. Interspecific crosses between *Vigna radiata* (MB) and *Vigna mungo* (BG)

Combinations Φ x Ơ	Pollination number	Fallen flowers number	Pod set number	Pod set percentage
MB × BG	134	69	65	48.5
BG × MB	94	74	20	21.3

Table 4. The number of embryos germinated in three types of media by *in vitro* culture

Media / Cultivar	MS				MS + 2BAP				MS + 4BAP			
Shiny green	4/5	5/5	5/5	4/5	4/5	5/5	5/5	4/5	3/5	4/5	2/5	0/5
Dark green	1/5	3/5	3/5	contaminate	1/5	contaminate	2/5	2/5	1/5	contaminate	0/5	0/5

4. Discussions

4.1. *In vivo* technique

In this technique, a comparison was done in terms of plants height. There was no any significant difference between the two cultivars in their height. However, SG05 showed higher height compared with DG04.

For the number of pods per plant, there were no significant differences. The average pod per plant for SG05 was 8 while DG04 had 6 pods in average. The highest and lowest number of pods per plant was 10 and 5 respectively in both cultivars. Inheritance of pods per plant is determined largely by additive gene action (Singh and Lal, 1975). Its expression is influenced by many genes including those controlling production, transport, and storage of assimilates; genes determining plant growth and development; and genes contributing to adaptation in stress environments.

There were slightly differences between SG05 and DG04 in terms of the number of seeds per pod. The average number of seeds per pod for SG05 was 10 compared to DG04 which was 9. The lowest and highest number of seeds per pod was 5 and 9 for both cultivars. Inheritance of seeds per pod is also determined largely by additive gene action (Wilson et al. 1985).

There were no significant differences in days to first flowering, plant height at flowering stage, days to first ripening, and nodes per plant. The days to first flowering of the two cultivars were 35 days after planting.

4.2. *In vitro* technique

The germination rate for SG05 was significantly higher than DG04. SG05 had the highest germination percentage, 75%, over DG04 which was just 29%. This was probably due to stress condition such as the sterilization procedure; incompatible media or stress condition within the culture and bottles.

The low germination percentage of DG04 was probably because of high concentration of Ethanol and other factors like exposure time and explant type that gave opposite effect. Mung bean seeds that were surface sterilized with 70% alcohol showed the reduction of survival percentage to 22%. This may be due to the toxic effect of alcohol that affects the cells activity or kills the embryo.

Nutrient medium is the second important parameter that must be optimized to obtain successful plant regeneration (Tisserat, 1985). Tisserat (1985) also stated that other factors like the genotype constitution, medium composition, culture environment, technique skill, and explant selection can affect the results. Each tissue type requires a different formulation depending on the objective of the study. Based on Table 4, MS media alone gave

the highest embryo germination compared with the MS media with 4 BAP. This was probably due to incompatible chemical substance such as high concentration of BAP that inhibited the proliferation of embryo.

There were no significant differences in the number of pods per plant between SG05 and DG04. The number of pods per plant was 3 for both cultivars which were considered as very low yield over the *in vivo* technique.

In terms of the number of seeds per pod, no significant differences were observed in the average number of seeds per pod between two cultivars. The average of 6 seeds per pod was obtained for two cultivars which were probably correlated with the growth retardation that affected the total yield of *in vitro* cultured Mung bean.

4.3. Comparison between *in vivo* and *in vitro* techniques

Results from this study revealed that *in vivo* technique gave the highest yield and yield components compared to the *in vitro* technique. This was probably because of the growth retardation of the *in vitro* procedure especially after acclimatization of Mung beans although it gave a significant increase in germination rate. Using the traditional methods, the total yield can be increased by natural planting.

4.4. Intraspecific hybridization

In most cases, pods abscised one week after pollinations, which may be due to failure of embryo development. Some pods developed normally to full length but dried prematurely and contained seeds of three kinds; empty, shriveled or normal but slightly crinkled. Some of the pods set developed normally to maturity but contained seeds which were small and crinkled and no normal seeds were found.

There were no initial barriers in the cross of *Vigna radiata* x *Vigna mungo* as the results showed a high percentage of pods set (48.5%). There were also no initial barriers in the cross of *Vigna mungo* x *Vigna radiata* as results showed percentage of pods set which was 21.3%. The production of viable and not viable seeds in these crosses, reciprocally, suggested that the barriers operated in the course of embryogenesis. The number of fertilized ovules in the mature pods was fairly high in these crosses and this suggested that incompatibility occurred between the embryo and its supporting systems like endosperm, suspensor, and maternal tissues.

The present study showed the existence of several isolating mechanisms operated between *Vigna radiata* and *Vigna mungo.* The isolating mechanisms seemed to be zygote mortality and hybrid unviability, which were due to abnormal embryo development. The crosses between these two species showed that the most compatible cross in relation to the percentage of pods set and normal mature seeds per pollination were *Vigna radiata* × *Vigna mungo* when *Vigna radiata* was used as female parents.

5. Conclusions

This study indicated that both SG05 and DG04 had the same agronomic potential by *in vivo* rather than *in vitro* technique. Short duration of plantings Mung bean and high germination percentage of seeds make it unsuitable for *in vitro* culture in a large scale for agriculture. Hybridization is the principal breeding procedure to combine agronomical useful traits, diseases resistance and good quality. So, the need for objectives that improve yield and stabilize production should be targeted. However, further research should be done in different cultivars and environments to select the suitable Mung bean for a specific region.

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