2. Malaysia

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Introduction

Orchid is one of the largest families of flowering plants in the world comprising of an estimated 20,000 to 25,000 species and untold number of hybrids of some 700 different genera. Some estimates have put the registered number of hybrids at more than 50,000 entries, with additional new ones at a rate of 1,000 per year. Orchids can be found growing at almost every latitude and stratosphere, where plants can normally grow. The range of different characteristics in shapes, sizes and colors have made orchids the plant for all types of interests and sought for by collectors.

The successes of hybridization technology in producing vast number of orchid hybrids with attractive characteristics and the introduction of the *in vitro* technology to mass-propagate clonal planting materials have been a tremendous boost for the orchid growing industry. Wide range of successful cultivars with attractive combinations of spray length, bud number, flower color and form, vase life, fragrance, seasonality, and compactness have been produced through hybridization. Meanwhile tissue culture has been the standard method of germinating seeds and propagating seedlings for the industry. Through the meristem cloning technique, thousands of plants can be grown in a relatively short period of time and every regenerated plant is exactly similar to the parent plant in every aspect. These two technologies have so far been very reliable in supporting the orchid industry in the region. Commercially attractive hybrids and varieties have been able to be mass propagated and supplied to the growers for the market.

Orchids such as *Dendrobium* have been the major orchid cut-flower export for Malaysia as well as for other Southeast Asian countries like Thailand and Philippines. Thailand is the major orchid producer with an export value of US$ 80 million in 2006, or approximately 80 % of the country's total ornamental export value (www.reedexpo.com/images/100266 /documents/OrchidPavilion.doc). The export value of orchids from Malaysia is estimated at

RM 150 million per year, representing approximately 40 % of the total floriculture production.

Problems regarding the infestation of insects in the orchid flowers have caused a lot of losses to exporters due to strict quarantine regulations. Presence of even one insect can cause the whole export consignment to be rejected by importing countries. There were cases when the whole orchid consignment had to be shipped back to the exporting countries due to the presence of insect pests. Spraying with insecticides and miticides are the options when dealing with orchid pests, especially when populations are high. This practice however leads to heavy use of chemicals but the problems with insects still persist. Studies have been conducted for post-harvest disinfestations of cut flower using irradiation. However, the post-harvest irradiation treatment has shown detrimental effect on the quality of the cut flowers. The strategy that can be adopted to overcome the problem is to breed for insect resistance in orchid. Insect resistant orchid hybrid may minimize the use of chemicals and overcome the strict quarantine requirements of importing countries.

Mutagenesis is considered as an appropriate approach to induce resistance as hybridization is limited by the unavailability of a resistant genotype and problem of sexual compatibility. Insect resistance has been induced by using mutagenesis approach in varieties of plants such as mungbean and rice. Mutation induction by irradiation has effectively changed certain characteristics of the plants to be 'unattractive' to insects.

Through biotechnological techniques, the insect resistance can be achieved via two approaches. The first used genes that synthesize products, which are toxic to feeding insects (eg Bt insecticidal protein and the cowpea trypsin-inhibitor protein) (Perlac et al. 1991; Hilder et al. 1987). The second approach used a cytokinin-synthesis gene 9ipt), which under the control of a specific promoter will induce the synthesis of one or more insecticidal metabolites (Smigocki et al. 1993). Several reports have shown that transformation of orchids is possible using the particle gun (biolistic) and the *Agrobacterium*-mediated method (Knapp et al. 2000; Wee et al 1999). Thus it is possible to introduce the genes for insect resistance into orchids.

Following consultations with the local orchid growers, we have identified that mites and thrips are the main pests for orchids in Malaysia. Other pests include aphids, whiteflies,

mealybugs, slugs and snails. At present, more than 30,000 mites have been identified all over the world. Most of them are parasitic on animals and humans, with only a few feeding on plants. Among the most common mites for orchids are false spider mites or flat mites (Gough, 1988). Flat spider mite or *Tenulpalpus orchidarum* is from the family of Tenuilpalpid. Flat mites are native to tropical and subtropical habitats and hosts, and are moved globally by the plant trade. Flat mites are very difficult to see without magnification although they move very slowly and they have a great resemblance with other mite species. Generally, adult flat spider mite can only reach up to 0.03 mm in length. These mites are pale yellowish-green to orange-red color. Flat spider mites have a flat appearance somewhat resembling spider mites and are often reddish colored with patterns of dark pigmentation.

Another common mite pest for orchids in Malaysia is *Tetranychus urticae* (twospotted spider mite). The pest is commonly found in the lower surface of leaves and sucks the sap of the leaf. The leaves become pale yellow with numerous small spot which reduces the quality of the leaves. The pest can be controlled with 0.03 % sulphex spray.

Mites normally feed by sucking the sap from individual cells on the surface of the leaves. Because of their feeding style, they may also be potential vectors for various diseases. Leaves infested by mites appear silvery, especially on the underside, where the cells of the surface layer were damaged. From the top, the leaf often has many tiny yellow spots. The false spider mites do not make silk, and are extremely tiny and hard to see, but they produce the same silvery structures on the leaves. A good way to know the presence of mites is to wipe the leaf with a white tissue; if the tissue has red smears, then the plant has mites.

Thrips is another important insect pest for orchids in many countries including Malaysia, and causes a lot of problems in flower production. Thrips normally infest young flower buds and newly expanded leaves. Blooms of infested plants may become prematurely brown, whilst the infested petals may either become spotted, streaked, silvery or discolored. Symptoms on leaves include chlorotic spots, wilting and eventually dropping. Plant growth can also be stunted, and in a severe infestation case, the whole plant will die (Jones 2008). Examples of mite and thrips infestation symptoms commonly found on infested orchid plants are shown in Figure 1.

Figure 1 Common infestation symptoms by mites (A) and thrips (B) on orchid plants

Three orchid species used in this project were *Dendrobium mirbellianum* (Malaysia), *Dendrobium* Sonia 'BOM 17 Red' (Thailand) and *Dendrobium jayakarta* (Indonesia). *Dendrobium mirbellianum* is a species commonly found in the Pacific region. It is easy to grow, robust and produces long spray (up to 45 cm) with up to 30 flowers. It is flowering throughout the year and the flowers last for about 4 weeks. Each flower measures between 3.5 - 5.5cm across. This species is lacking in several qualities such as attractive color and long lasting shelf life, thus making it as a good candidate for the mutagenesis project.

Dendrobium Sonia 'BOM 17 Red' is a very popular commercial hybrid in the region and widely grown for the cut flower export market. This hybrid has purple flowers that can last up to 14 days as cut flower. This commercial hybrid was chosen as it is one of the most popular cut flower *Dendrobium* hybrids grown in the country. Several variations of this hybrid differing in petal and sepal pigmentation are available such as Sonia and Sonia BOM Jo. Meanwhile, *Den. jayakarta* is a popular commercial hybrid in Indonesia with white colored flowers. Figure 2 shows the three orchid species used in this project.

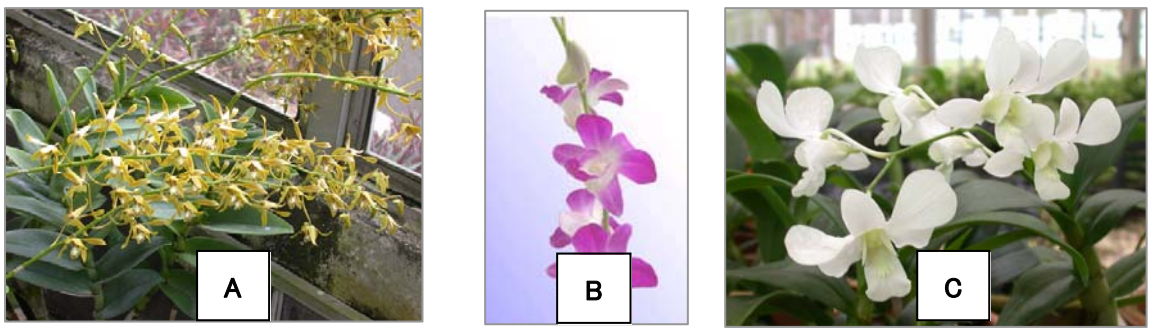

Figure 2 : Orchid species used in the project;
***Den. mirbellianum* (A), *Den.* Sonia 'BOM 17 Red' (B) and *Den. jayakarta* (C)**

Objective of the Project

The main objective of this project is to produce orchids which are resistant / tolerant to insect pests, mainly mites and thrips.

Approach and Technology

In this project three main technologies were employed and developed. They were:

1. Mutagenesis using gamma irradiation and ion beam - Gamma and ion beam irradiation technology were used to generate mutant lines with potential resistant to insects.
2. Selection of insect resistant orchids - The techniques of screening were developed in this study to select plants resistant to insect infestation at both *in vitro* (tissue culture) as well as *in vivo* (flowering) stages.
3. *Agrobacterium*-mediated genetic transformation was also used and optimized to transfer genes that may confer insect resistance in *Dendrobium* species/hybrids used in this study.

Materials and Methods

Plant materials

Irradiated PLBs of *Dendrobium* Sonia 'BOM 17 Red' were obtained from Thailand (courtesy of Associate Professor Dr. Chitrapan Piluek, Kasetsart University, Bangkok), whilst non-irradiated PLBs of *Den. jayakarta* was obtained from Indonesia (courtesy of Ms Ismiyati Sutarto, BATAN, Indonesia). For *Den. mirbellianum,* PLBs were initiated from mature seeds of self-pollinated flowers. The seed capsules were surface sterilized by dipping them in 90 % ethanol and flaming. They were cut open with sterile tools and the seeds were germinated on half-strength (½ MS) medium (Murashige and Skoog, 1962) at 26 °C with 16h photoperiod until PLBs were formed. These PLBs were maintained in culture on ½ MS medium at the same temperature and photoperiod, and subcultured every 4 weeks on fresh media.

Ion beam irradiation

PLBs of *Den. mirbellianum* and *Den. jayakarta* that were uniform in size were transferred into sterile 6.0 cm Petri dish, covered with a sterile 8 µm-thick polyimide film (Kapton^{R} Du Pont-Toray, Japan) and sealed with Nescofilm. These PLBs were then irradiated with 220MeV $^{12}C^{6+}$ ion beam at Takasaki Ion Accelerators for Advanced Radiation (TIARA), JAEA, Takasaki at doses 0, 0.4, 0.8, 1.0 and 2.0 Gy for *Den. mirbellianum* and 0, 1.0 Gy and 2.0 Gy for *Den. jayakarta*. Two to three days after irradiation, PLBs were transferred onto fresh ½ MS medium.

The PLBs were allowed to proliferate and multiply by subculturing onto fresh medium every four weeks. After four periodic subcultures, the irradiated PLBs were allowed to regenerate into whole plantlets, transferred into pots and grown to maturity until flowering. For *in vitro* insect resistance study, plantlets were placed individually in culture vials.

Gamma irradiation

Dendrobium mirbellianum

Den. mirbellianum plantlets with 3 to 4 expanded leaves and almost similar in size were placed on petri dishes containing ½ MS Agar. Each petri dish contains 5 plantlets. They were irradiated at doses of 0, 20, 40, 60, 80, 160, 320, 640 and 1,280 Gy using a gamma chamber (JL Shepherd). Irradiated plantlets were subsequently removed from agar, cleaned under running tap water and transferred onto wet tissue in the lab for one week to allow the plantlets to be hardened before being planted in the nursery. Assessment on the survival rate of the plantlets was done every week for 4 consecutive weeks.

Gamma chamber in Malaysia

Dendrobium Sonia 'BOM 17 Red'

PLBs of *Den.* Sonia 'BOM 17 Red' from Thailand have been irradiated with gamma ray at doses of 0, 60, 70, 80 and 90 Gy .The PLBs were multiplied, regenerated into complete plantlets, and transplanted in the glasshouse.

Dendrobium '*Jayakarta*'

Irradiation of *Den. jayakarta* PLBs using gamma rays (JL Shepherd) was carried out using two approaches;

i. A single dose of 30 Gy (acute radiation)
ii. Fragmented dose (10 Gy every week for 3 weeks)

The irradiated PLBs were cultured on ½ MS agar. Surviving PLBs were multiplied on the same medium until the fifth generation. After five periodic subcultures, the irradiated PLBs were allowed to regenerate into whole plantlets and transferred to glasshouse.

Mites culturing / rearing

Initially, mites were propagated in petri dishes containing clean *Arachis pintoi* leaves and wet cotton, to study their morphology and reproductive behavior. They were segregated according to their life stages; adult, deutonymph, protonymph, larvae and egg. Segregation according to sex was done to adult mites. Female and male adults were allowed to mate and the duration of the development of different life stages produced were monitored and recorded.

The mites used for screening of irradiated orchid plantlets were later reared and multiplied *in vitro*. Adult mites (*Tenuipalpus pacificus*) collected from nursery-grown orchid plants were placed in tissue culture jars containing *Den. mirbellianum* plants having at least two open leaves. The jars were subsequently placed at room temperature (approximately under 10 hours photoperiod), under rearing temperature at 27 °C and approximately 70 % relative humidity to allow the mites to multiply (Krantz 1978). Adult mites were transferred to new tissue cultured *Den. mirbellianum* plants after 2 weeks

***In vitro* mites infestation and screening**

Irradiated orchid plantlets used for *in vitro* screening were put in individual vials. Plantlets of about 4 cm with 4 open leaves were used in this study. Three female and two male adult mites were then put in the vials containing the plantlets. Observations on the pattern of infestation and the multiplication of mites were made every week according to Zhang (2001). Plantlets showing no or less symptom of infestation will be isolated and mass propagated.

For symptom analysis, each leaf was divided into five regions (Figure 1). Infestation was detected using a dissecting microscope. Scoring was done for the first 3 consecutive weeks and after 9 weeks, by estimating the area of infestation as shown in Figure 1. The infestation levels were scored as follow; 1: no infestation, 2: less than 5 % infestation, 3: 5 - 10 % infestation, 4: 11 - 25 % infestation and 5: more than 25 % infestation. In cases where the infestation occurred on different leaves, the areas of infestation were combined to get the total infestation area.

After 3 months of infestation, surviving plants were transferred into small pots for hardening and left to grow in the greenhouse until flowering. Fertilizers were applied to the plants for growth, but not pesticides. Secondary screening at the flowering stage by infestation with the target insects were carried out on these potential mutants.

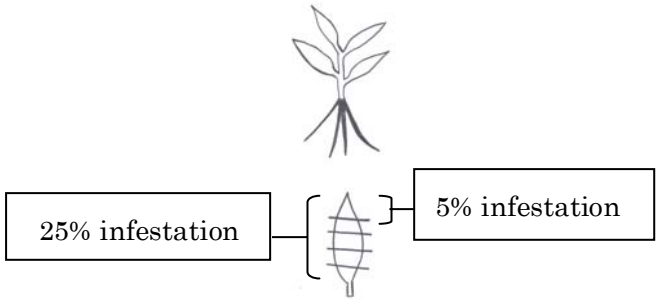

Figure 1 Illustrated scoring index for mite infestation on orchid leaf

***In vitro* thrips infestation and screening**

In vitro screening on irradiated orchids for tolerance towards thrips was carried out using the same procedure for mite screening (as discussed above). Thrips were collected directly from heavily infested orchid plants.

***In vivo* thrips infestation**

In vivo thrips infestation studies were carried out on flowering plants in a nursery dedicated for insect rearing and screening. Flowering plants of potential insect tolerant mutants identified at tissue culture stage, as well as randomly selected irradiated plants were placed alongside heavily thrip-infested plants in the nursery. Observations on the flowers were made for 30 days from the date of infestation. Non-irradiated plants were used as controls.

Orchid transformation and regeneration

Genetic transformation work has been initiated on *Den. mirbellinium* in 2005, using *Agrobacterium tumefaciens* LBA4404 strains, carrying plasmid with a marker gene (hygromycin resistance), a reporter gene (GUS) and a chitinase gene. The *Agrobacterium* was maintained on LB agar plate containing 100 mg/L kanamycin. A single colony was inoculated overnight in liquid LB medium at 28 °C with 100 mg/L kanamycin and 200 uM acetosyringone. For transformation, the bacterial pellet was diluted in MS liquid in a ratio of 1:10. Protocorm-like bodies (PLBs) of *Dendrobium mirbellianum* were generated as described above.

PLBs were pre-cultured for 5 days on ½ MS agar-solidified medium and incubated at 25 °C ± 2 with 16 hours photoperiod. Prior to inoculation, 1 ml of overnight grown *Agrobacterium* was centrifuged at 5,000 g and the pellet was dissolved in 10 ml ½ MS liquid medium (1:10 dilution). PLBs were inoculated by immersing in the diluted bacterial solution for 30 minutes

with occasional shaking, blot dried and were placed back on ½ MS agar-solidified medium. The inoculated PLBs were co-cultivated at 25 ± 2 °C in the dark for 3 days to allow the transfer of genes from *Agrobacterium* into plant cells.

Following 3 days of co-cultivation, the PLBs were washed with sterile water and transferred into conical flasks containing ½ MS liquid medium supplemented with 100 mg/L cefotaxime. The flasks containing PLBs were placed on an orbital shaker for 4 hours to remove traces of *Agrobacterium.* Subsequently, the PLBs were collected and blotted dry on sterile tissue paper before being placed on ½ MS agar-solidified medium containing 100 mg/L cefotaxime for a week. After one week, the PLBs were transferred again to ½ MS agar-solidified medium containing 10 mg/L hygromycin for selection. Surviving PLBs were subcultured onto fresh selective medium every 4 weeks until the formation of plantlets.

Results and Discussion

Orchid tissue culture

Plantlets of all varieties were successfully regenerated and planted in the glasshouse. Of these, *Den. mirbellianum* and *Den. Jayakarta* plants have been flowering, whilst *Den.* Sonia 'BOM 17 Red' plants are still in growing stage and have not flowered.

Ion beam irradiation

Initial radiosensitivity data has shown that the optimum dose for ion beam irradiation of orchid PLBs was in the range of 0.8 to 1.0 Gy (Sakinah et al. 2005). This data was obtained through studies on orchid mutagenesis using ion beams under bilateral project between Nuclear Malaysia and JAEA. Therefore, for *Den. mirbellianum*, PLBs irradiated at doses of 0.4, 0.8, 1.0 and 2.0 Gy were used for this project, whilst those from other doses will be used for *in vivo* screening of other characteristics such as flower color and morphology. For ion beam irradiated *Den. jayakarta*, a number of plantlets from all doses (0, 1 and 2 Gy) were also infested with mites and thrips.

Gamma irradiation

Gamma irradiation of *Den. mirbellianum* plantlets showed that the plantlets were sensitive gamma ray above 600 Gy. Most plantlets irradiated below 640 Gy were still viable after 1 week. However after four weeks, those irradiated at higher doses could not survive; i.e only 47.5 % plantlets at 640 Gy were viable and only 2.5% at 1,260 Gy. Table 1 shows detailed

results on gamma irradiation of *Den. mirbellianum* plantlets. Gamma irradiated *Den.* Sonia 'BOM 17 Red' and *Den. jayakarta* were also been planted in the glasshouse and used for *in vivo* screening of insects.

Table 1. Effect of gamma irradiation on the viability of *Den. mirbellianum* plantlets

Dose (Gy)	Viability after 1 week		Viability after 4 weeks	
	No. of viable plantlets	%	No. of viable plantlets	%
0	40	100	38	95
20	37	92.5	37	92.5
40	37	92.5	37	92.5
60	38	95	34	85
80	37	92.5	27	67.5
160	38	95	32	80
320	33	82.5	27	67.5
640	30	75	19	47.5
1280	7	17.5	1	2.5

Note: The number of replicates per dose were 40

Mites culturing and rearing

Work on multiplying adult mites in large number has been carried out. It was observed that the complete life cycle of mites (from adults to adults) under Malaysian weather was approximately 9 weeks. They favour warm to hot dry conditions to multiply. Under normal environment, a female mite would produce approximately 5 eggs per day. In this study, of the five mites used to inoculate an *in vitro* orchid plant, two were male mites. Male mites were morphologically smaller than female mites.

***In vitro* screening for mite tolerance**

In vitro screening on ion beam irradiated *Den. mirbellianum* plantlets, has shown that after one week of inoculation with mites, majority of the tested plants from all doses of irradiation, showed a low level of infestation. Plants irradiated at doses above 1 Gy in particular, only showed less than 5% infestation (Table 2). In week 2 and 3, more infestation on the plants'

leaves was observed, especially those irradiated at doses below 0.8 Gy (Table 3 and Table 4). However, there was no significant difference in the number of surviving adult mites, the number of eggs and the number of larvae produced after 3 weeks of inoculation on *in vitro* plants. This suggested that the irradiated plants did not produce toxin that can kill mites, but rather develop internal resistance to mite infestation. Table 5, 6 and 7 show detailed results on the number of surviving adult mites, eggs and larvae produced on the irradiated plants tested, respectively.

Table 2. Severity of infestation after 1 week of inoculation with mites

Dose (Gy)	# of plants	No. of plants at different infestation scale (%)				
		1	2	3	4	5
0	52	43 (82.69)	4 (7.69)	5 (9.61)	0	0
0.4	50	44 (88.0)	5 (10.0)	1 (2.0)	0	0
0.8	57	51 (89.47)	4 (7.02)	2 (3.51)	0	0
1.0	26	26 (100)	0	0	0	0
2.0	10	9 (90.0)	1 (10.0)	0	0	0

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves, 4 : 11-25% infested leaves
5 : > 25% infested leaves

Table 3. Severity of infestation after 2 weeks of inoculation with mites

Dose (Gy)	# of plants	No. of plants at different infestation scale (%)				
		1	2	3	4	5
0	52	33 (63.46)	6 (11.54)	7 (13.46)	5 (9.62)	1 (1.92)
0.4	50	38 (76.0)	1 (2.0)	7 (14.0)	4 (8.0)	0
0.8	57	47 (82.46)	3 (5.26)	2 (3.51)	5 (8.77)	0
1.0	26	21 (80.77)	4 (15.38)	1 (3.85)	0	0
2.0	10	7 (70.0)	2 (20.0)	1 (10.0)	0	0

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves, 4 : 11-25% infested leaves,
5 : > 25% infested leaves

Table 4. Severity of infestation after 3 weeks of inoculation with mites

Dose (Gy)	# of plants	No. of plants at different infestation scale (%)				
		1	2	3	4	5
0	52	33 (63.46)	4 (7.69)	5 (9.62)	8 (15.38)	2 (3.85)
0.4	50	37 (74.0)	2 (4.0)	3 (6.0)	3 (6.0)	5 (10.0)
0.8	57	45 (78.95)	3 (5.26)	3 (5.26)	4 (7.02)	2 (3.51)
1.0	26	21 (80.77)	0	4 (15.38)	1 (3.85)	0
2.0	10	7 (70.0)	1 (10.0)	2 (20.0)	0	0

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves 4 : 11-25% infested leaves, 5 : > 25% infested leaves

Table 5. Number of surviving adult mites after inoculation on *in vitro Den. mirbellianum* orchids

Dose (Gy)	# of inoculated mites (initial)	# of Adult Mites (%)		
		Week 1	Week 2	Week 3
0	260	166 (63.85)	162 (62.31)	165 (63.46)
0.4	250	147 (58.8)	146 (58.4)	141 (56.4)
0.8	285	184 (64.56)	182 (63.86)	176 (61.75)
1	130	70 (53.85)	68 (52.31)	69 (53.08)
2	50	32 (64.0)	32 (64.0)	32 (64.0)

Table 6. Number of eggs after inoculation on *in vitro Den. mirbellianum* orchids

Dose (Gy)	# of inoculated mites (initial)	# of Eggs (%)		
		Week 1	Week 2	Week 3
0	260	93 (35.77)	116 (44.62)	122 (46.92)
0.4	250	87 (34.8)	92 (36.8)	102 (40.8)
0.8	285	54 (18.95)	124 (43.51)	114 (40.0)
1	130	61 (46.92)	68 (52.30)	63 (48.46)
2	50	31 (62.0)	34 (68.0)	34 (68.0)

Table 7. Number of larvae after inoculation on *in vitro Den. mirbellianum* orchids

Dose (Gy)	# of inoculated mites (initial)	# of Larvae (%)		
		Week 1	Week 2	Week 3
0	260	1 (0.38)	13 (5.0)	2 (0.77)
0.4	250	0	6 (2.4)	3 (1.2)
0.8	285	0	2 (0.7)	8 (2.81)
1	130	0	2 (1.54)	4 (3.08)
2	50	0	0	2 (4.0)

The severity of infestation on the infested *Den. mirbellianum* plantlets was monitored for 9 weeks, or until a complete life cycle of mites (Table 8). At this stage, it was also observed that all non-irradiated plants tested showed a minimum 5 % of the infestation symptom on their leaves. Some of these control plantlets were also severely damaged and died. Irradiated plantlets did also exhibit infestation symptoms at various scales, ranging from very mild to heavily infested, but there were also plantlets that showed no sign of infestation and completely healthy. The percentages of non-infested plantlets were gradually increased with the increase in treated irradiation doses. The percentages of non-infested plantlets were 34 %, 24.56 %, 50 % and 60 % for those irradiated at 0.4, 0.8, 1.0 and 2.0 Gy, respectively. These results showed an increase in resistance towards mite infestation on irradiated seedlings.

Table 8. Severity of infestation after 9 weeks of infestation with mites

Dose (Gy)	# of plants	No. of plants at different infestation scale (%)				
		1	2	3	4	5
0	52	0 (0)	0 (0)	0 (0)	9 (17.31)	43 (82.69)
0.4	50	17 (34.0)	5 (10.0)	2 (4.0)	1 (2.0))	25 (50.0)
0.8	57	14 (24.56)	2 (3.51)	1 (1.75)	6 (10.53)	34 (59.65)
1.0	26	13 (50.0)	2 (7.69)	2 (7.69)	2 (7.69)	7 (26.92)
2.0	10	6 (60.0)	1 (10.0)	1 (10.0)	1 (10.0)	1 (10.0)

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves 4 : 11-25% infested leaves,
5 : > 25% infested leaves

Overall, 50 potential *Den. mirbellianum* mutants were identified as tolerant to mite infestation and successfully planted in the glasshouse. Of these, 11 plants were from 0.4 Gy, 31 from 0.8 Gy and 8 from 1.0 Gy. These plants were further propagated in the glasshouse for subsequent screening at flowering stage. Figure 3 shows the chronology for producing mite tolerant *Den. mirbellianum.*

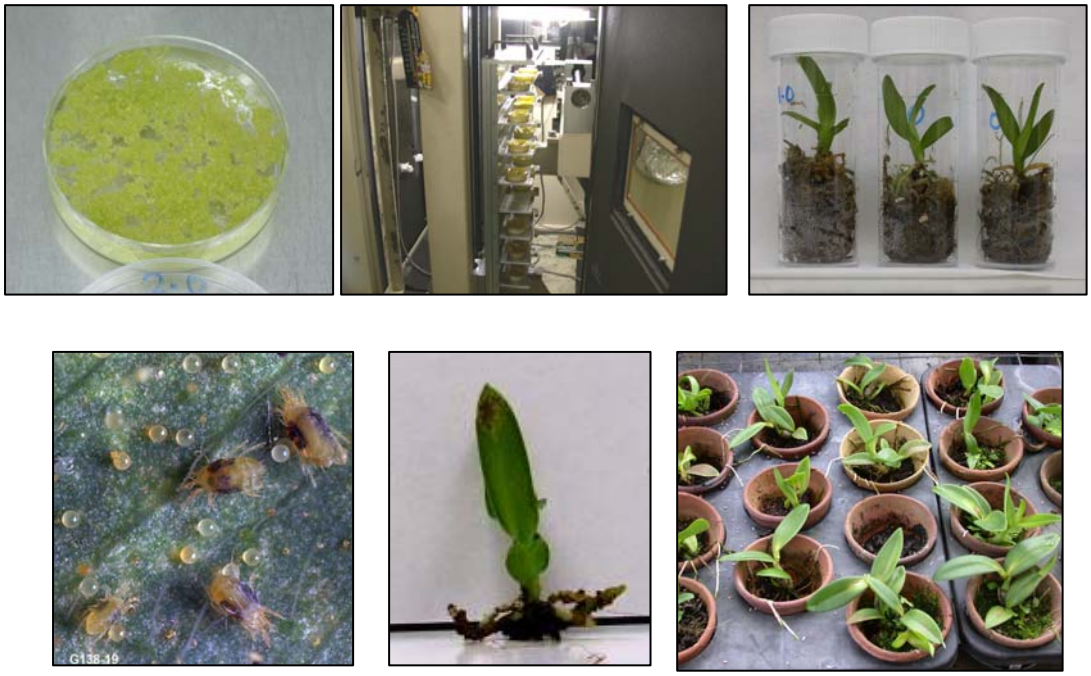

Figure 3. Development of potential mite resistant *Den. mirbellianum* mutants

A: Orchids PLBs used for irradiation B: Ion beam irradiation facility, JAEA, Japan

C: Individual plantlets in vials for in vitro challenge-infestation

D: Infesting pest (mites) E: Symptom of infestation on orchid leaf

F: Potential mite resistant orchid mutants in glasshouse

***In vitro* thrips infestation**

Thrips have been known to cause injury to leaves and buds in infested orchid plants. Among the symptoms observed are browning or blotching of the leaves and bud destruction (http:www.ct.gov/caes/cwp/view.asp). Another symptom is severe flower break as a result of virus infestation transmitted by thrips (Pataky 1990).

An experiment was carried out to screen *in vitro* irradiated plantlets against thrips. Due to a limited number of thrips, only a small number of irradiated plants were used in this experiment. Table 9 and Table 10 show detailed results on the number of infested plants and the severity of infestation with thrips, after week one and two, respectively.

Based on this work, thrips were seen to cause rapid and severe damages on the infested plants as compared to mites. After only 2 weeks of infestation, all control or non-irradiated plants showed symptoms of infestation. Of these, 100 % of *Den. mirbellianum* and 83.3 % of *Den. jayakarta*, exhibited severe damage on more than 25 % of their leaf areas. A number of irradiated *Den. jayakarta* and *Den. mirbellianum* plantlets displayed early tolerance to these infestations. Overall from the *in vitro* screening, 2 *Den. mirbellianum* and 5 *Den. jayakarta* plants were selected as potential tolerant to thrips. Figure 4 shows some infestation symptoms on *in vitro* plantlets infested with thrips.

Table 9. Severity of infestation after one week of infestation with thrips

Orchid species **Dose (Gy)**	**# of plants**	**No. of Infested plants**				
		1	**2**	**3**	**4**	**5**
Den. mirbellianum (gamma irradiated)						
0	5	3	2	0	0	0
0.4	5	5	0	0	0	0
0.8	5	3	2	0	0	0
1.0	5	5	0	0	0	0
Den. jayakarta (gamma irradiated)						
0	6	1	1	4	0	0
10	5	5	0	0	0	0
30	5	4	1	0	0	0
Den. jayakarta (ion beam)						
1.0	5	3	1	1	0	0
2.0	5	2	3	0	0	0

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves 4 : 11-25% infested leaves,
5 : > 25% infested leaves

Table 10. Severity of infestation after 2 weeks of infestation with thrips

Orchid species Dose (Gy)	# of plants	No. of Infested plants				
		1	2	3	4	5
Den. mirbellianum (ion beams)						
0	5	0	0	0	0	5
0.4	5	2	2	1	0	0
0.8	5	0	2	0	3	0
1.0	5	0	3	0	2	0
Den. jayakarta (gamma irradiated)						
0	6	0	1	0	0	5
10	5	2	0	1	0	2
30	5	1	1	1	0	2
Den. jayakarta (ion beam)						
1.0	5	1	1	0	1	2
2.0	5	1	0	1	1	2

Note; 1 : no infestation, 2 : < 5% infested leaves, 3 : 5-10% infested leaves 4 : 11-25% infested leaves,
5 : > 25% infested leaves

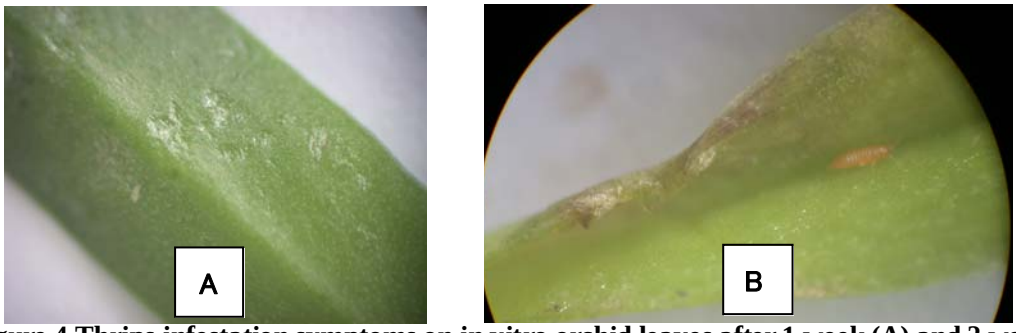

Figure 4.Thrips infestation symptoms on in vitro orchid leaves after 1 week (A) and 2 weeks (B)

<u>Potential mutant lines from *in vitro* screening</u>

Den. mirbellianum

Based on *in vitro* mite screening tests, 50 *Den. mirbelliannum* lines, which were irradiated with ion beams, have been identified as potential mite tolerant mutants. These lines were already planted in the glasshouse. Of these, 11 plants are from 0.4 Gy, 31 from 0.8 Gy and 8 from 1.0 Gy. From *in vitro* thrips screening, 2 plants were found tolerant.

Den. jayakarta

Based on *in vitro* thrips screening, 3 *Den. jayakarta* plants irradiated with gamma rays were identified as potential thrip tolerant, whereas 2 plants irradiated with ion beams were tolerant.

<u>*In vivo* thrips screening</u>

After 3 months of infestation, healthy and surviving plants were transferred into small pots for hardening. Subsequently they were allowed to grow under shade in the greenhouse. The plants were regularly sprayed with fertilizers for growth but were not sprayed with any pesticides. In nature, expressions of disease or infestation symptoms in plants are influenced by a combination of factors such as susceptibility of the host, infective pathogen and favourable environmental conditions (Singh & Singh, 2005). Therefore, these potential mutant plants were also screened at flowering stage by challenge-infestation with the target insects, since insects like thrips especially feed on flowers. It was also a way to confirm the stability of the trait in natural growing conditions.

To date, the tests were carried out on irradiated *Den. jayakarta* plants and potential mite resistant mutants of *Den. mirbellianum* only, since *Den.* Sonia 'BOM 17 Red' have not flowered yet. Infestation severity index was developed based on the level of damages caused by thrips to these flowers. The scale was between 1 and 5, where 1 was resistant and 5 was totally susceptible (Figure 5).

Based on the *in vivo* thrips screening, all samples of *Den. jayakarta* plants from the control and a fragmented dose (gamma, 3 x 10 Gy) populations, were found to be susceptible to insects. Two lines from a single dose (gamma, 30 Gy) population exhibited minor damages (scale 2) on their flowers, and thus could be considered potential resistant lines. These plants have been tagged and maintained in the glasshouse.

For mite tolerant *Den. mirbellianum* plants, the same index was applied and one plant was found tolerant to thrips. All the thrips and mite tolerant mutants are now being propagated in the nursery, to achieve large number of clones.

Genetic transformation

The objective of this genetic transformation work was to study the possibility of generating insect resistant plants through the insertion (transformation) of chitinase gene. From the experiments on *Den. mirbellianum* using *A. tumefaciens* carrying a chitinase gene, 5.59% of the putatively transformed PLBs were obtained after the first selection on hygromycin selection media. These PLBs were transferred to fresh selection media to let them to proliferate. The PLB-to plant conversion was also carried on the same selection media. A total of 13 hygromycin resistant plants have been successfully achieved. At present, the plants are being maintained, and further screening and evaluation will be carried out in the transgenic facility, which is currently under construction.

Conclusion

Some observations and findings of this project were;

1. The optimum ion beam irradiation dose for PLBs was 0.8 - 1.0 Gy.
2. The method for *in vitro* rearing of mites was established and from this study, the complete life cycle of mites in Malaysian conditions was found to be 9 weeks.
3. An *in vitro* insect infestation procedure was developed for preliminary selection of insect tolerant mutants at tissue culture stage.
4. *In vitro* mite infestation study on regenerated *Den. mirbellianum* orchids has showed an increase in resistance towards mite infestation on ion beam irradiated plantlets. The percentages of mite resistant mutant plantlets gradually increased with increasing irradiation doses.
5. Through *in vitro* mite infestation study, a total of 50 potential mite tolerant plantlets

were identified. Of these 50 plants, one plant was found tolerant to thrips when secondary screening was carried out at flowering stage (*in vivo*).

6. Through *in vitro* infestation with thrips, 2 *Den. mirbellianum* plantlets and 5 *Den. jayakarta* plantlets were tolerant. These plants are being propagated in the glasshouse, and secondary screening for thrip tolerance will be carried out once the plants are flowered.
7. *In vivo* thrip screening at flowering stage have identified 2 clones of *Den. jayakarta* which were tolerant to this insect.
8. All identified mutants are currently being propagated to achieve large numbers of clones, and will be transferred to a private collaborator for pre-commercialization studies.
9. Genetic transformation study on *Den. mirbellianum* has generated a number of putative transgenic plants which will be screened and evaluated in the transgenic facility.

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