MUTATION INDUCTION OF ORCHIDS BY ION BEAMS

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Abstract

Mutation induction using ionizing radiation provides an effective alternative means for improvement of orchids. In this study, ion beams were used because they have much higher linear energy transfer (LET) than X-rays or gamma rays, and subsequently lead to higher mutation frequency and broad mutation spectrum. The protocorm-like bodies (PLBs) of three orchid species (Dendrobium crumenatum, Dendrobium mirbellianum) were irradiated at various doses with 320 MeV $^{12}C^{6+}$ *ions accelerated by Azimuthally Varying Field (AVF) cyclotron at JAEA's Takasaki Ion Accelerators for Advanced Radiation Application (TIARA). The optimum irradiation condition and the effect of irradiation on each species were studied, particularly on flower colour and morphology, flowering habit and insect resistance. Dose effects on plantlet regeneration for each species were also obtained. Some morphological changes were observed in flowers of Dendrobium crumenatum, whilst one insect resistant mutant was obtained in Dendrobium mirbellianum.*

Keywords/Kata kunci: *Dendrobium sp.*, ion beams, mutation, flower morphology

INTRODUCTION

The technology for induction of mutations is a powerful tool for developing better varieties of food and industrial crops. Gamma irradiation and chemical mutagenesis are general methods for mutation induction and has been applied for plant breeding in many countries in the world. Whereas, establishing a new method has been expected to enhance more genetic diversity in available plant resources. Studies in JAEA have suggested that ion beams are characteristic of high relative biological effectiveness for survival and other endpoints, cause high mutation frequency, and are useful to isolate novel mutants in Arabidopsis (Hase *et al.*, 2002, Shikazono *et al.*, 2003 and Oono *et al.*, 2006). Mutation induction in chrysanthemums and carnations using ionizing radiation has also shown its reliability for the production of a wide range of variations with attractive combinations of spray length, bud number, flower color and form (Nagatomi *et al.*, 1996 and Okamura *et al.*, 2003). The successful results in chrysanthemums and carnations prompt us to apply ion beams to other plant species to generate novel varieties that have never been obtained by previously available methods.

Orchid industry is part of the global floriculture commerce valued at USD9 billion. In spite of its small share of fresh flower market, the orchid industry in Malaysia is developing into a very viable and lucrative commercial enterprise. Orchid growing in Malaysia is a multi-million ringgit industry, most notably in the cut-flower trade (Mohd. Khairol and Noor Auni, 1991). The value of Malaysia annual export and local markets is about RM40 million and RM20 million, both on flowers as well as plants (Guus Wijchman, 2005). Approximately 24.3 million stalks of orchid cut-flowers were produced in year 2000 with Dendrobium topping the list at 13.1 million stalks (FAMA, 2000). Its growth is expected to escalate since the government has given top priority to agriculture and export-oriented high-value products like orchids.

A major issue faced by the orchid industry in Malaysia is associated with the lack of varieties. In order to keep up with the ever-changing tastes of consumers, there is an urgent need to create new and better varieties of orchids to sustain the floriculture industry. The use of conventional breeding methods to create variation in orchids is restricted by sexual incompatibility, sterility problems and long breeding time.

Gamma irradiation has been successful in creating many *Dendrobium* 'Sonia' mutant varieties. In *Dendrobium* Ekapol and *Dendrobium* Sonia for examples, irradiation resulted in changes of flower pigmentation and size (Mohd Nazir *et al.*, 2001 and Sakinah *et al.*, 2002). More variations with attractive combinations of spray length, bud number, flower color and form are required to create commercially valuable varieties. Considering successful results in Arabidopsis, chrysanthemums and carnations, ion beams are tools to conduct orchid improvement.

The possibility of the application of ion beams to produce novel orchid varieties is investigated under the cooperative research program between Nuclear Malaysia and JAEA. The objectives are to develop a protocol for ion beam irradiation in orchids and to produce orchids with improved characteristic such as attractive flower colour and morphology, longer shelf life and good flowering habit.

MATERIALS AND METHODOLOGY

Plant materials

Two orchid species used in this project were *Dendrobium mirbellianum* and *Dendrobium crumenatum* (Figure 1). *D.mirbellianum* is a robust and easy-to-grown species which produces long spray (up to 45 cm) with up to 30 flowers. It has good flowering habit and the flowers can last for about 4 weeks. *D.crumenatum* is known as Pigeon orchid and easily cultivated. It grows rapidly with white fragrant flowers that only last for a day. The flowering of *D.crumenatum* is triggered by sudden drop in temperature.

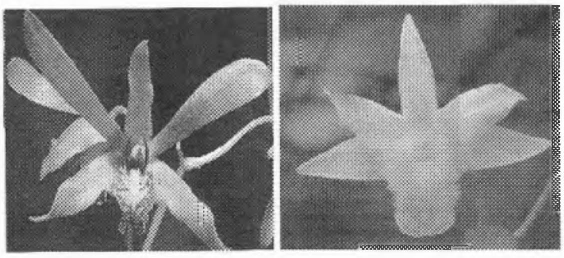

Figure 1. Flowers of *Dendrobium mirbellianum* (left) and *Dendrobium crumenatum* (right)

In vitro cultures of these species were established at Plant Biotechnology Laboratory, Malaysia Nuclear Agency. Mature seeds of these species were collected from self-pollinated flowers. The seed capsules were surface-sterilized by dipping them in ethanol followed by short flaming. They were cut open under a sterile condition and the seeds were germinated on half-strength Murashige and Skoog Media (½MS) (Murashige and Skoog, 1962) at 25±2 °C with 12-hour photoperiod until protocorm-like bodies (PLBs) were formed. PLBs that were uniform in size with approximately 2 mm in diameter were chosen for ion beam irradiation experiment. Figure 2 shows the state of PLBs used in the irradiation.

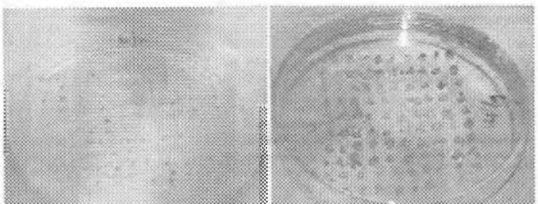

Figure 2. The state of PLBs for irradiation.

Ion beam irradiation

Irradiation was carried out at Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), Japan Atomic Energy Agency at Takasaki. PLBs were placed on 6-cm sterile petri dishes containing ½ MS medium and covered with a sterile 8 μm-thick plyimide film (Kapton® Toray, Japan). These PLBs were irradiated with 320 MeV $^{12}C^{6+}$ ions accelerated by Azimuthally Varying Field (AVF) cyclotron (Figure 3). The irradiated PLBs were brought back to Nuclear Malaysia for *in vitro* propagation and screening.

In the preliminary stage of this project, information on the effective dose for mutation induction in *Dendrobium* sp. has yet established. Therefore doses ranging up to 50 Gy (0, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 12, 15, 20, 30 and 50 Gy) were applied to the PLBs. It was found from this preliminary experiment that doses higher than 15 Gy totally inhibited growth in *D. mirbellianum*. Hence, doses less than 15 Gy were used in the subsequent experiments. The doses were 0, 0.2, 0.4, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, and 15.0 Gy.

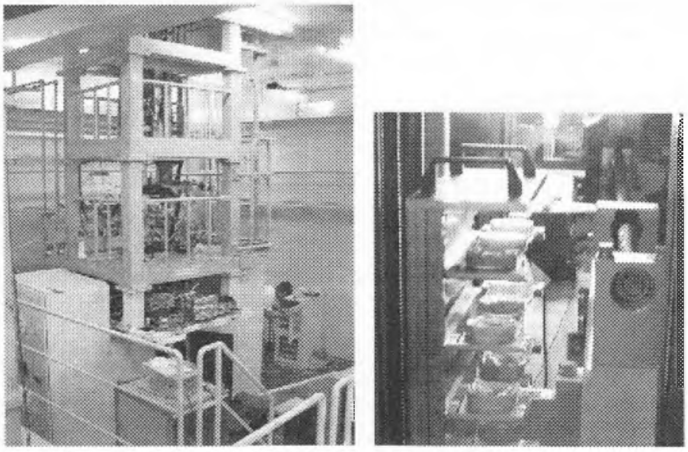

Figure 3. Irradiation with 320 MeV $^{12}C^{6+}$ ions accelerated by AVF Cyclotron at TIARA (right), aluminium plates with petri dishes are inserted in the irradiation apparatus (left)

Propagation and Screening

Propagation and screening of irradiated PLBs were carried out at Malaysian Nuclear Agency. The irradiated PLBs were transferred onto fresh ½ MS medium and incubated at 25±2 °C under 12-hour photo period for proliferation. Subsequently, the cultures were transferred onto fresh media every four weeks for multiplication and regeneration. The number of PLB that regenerates shoots was recorded after 2 months. Plantlets were allowed to proliferate and multiply for several months before being hardened in glasshouse.

RESULTS

Irradiation condition

The size of PLBs used for irradiation was found to be one of the important factors that influenced the survival of these PLBs after irradiation. PLBs which were less than 2 mm size could not survive the irradiation even at lower doses (0-1.0 Gy) as observed in cultures of *D.crumenatum* (Figure 4). The PLB were also needed to be precultured in the 6 cm petri dish for a week to allow them to stabilize under the culture conditions.

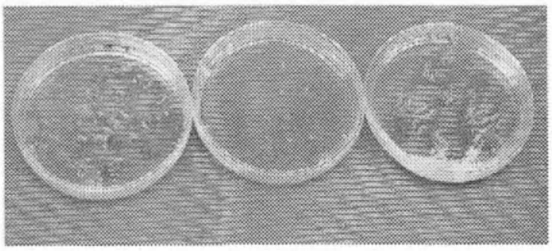

Figure 4. *D crumenatum* PLBs of size less than 2 mm irradiated at 10 Gy, 1 Gy and 4 Gy (from left to right).

Figure 5 and 6 show the relationship between doses (Gy) and percentage of regenerated shoots in *D mirbellianum* and *D.crumenatum,* respectively, which are recorded at 8[th] week. In *D.mirbellianum* culture, we were able to obtain regenerated shoots from 72% of PLBs of control or non-irradiated population. Effect of ion-beam irradiation was clearly observed as regeneration frequencies in the population irradiated at doses higher than 2 Gy were gradually reduced. Irradiation effect was reached to maximum at 6 Gy, where only 8% of PLBs were regenerated.

In *D.crumenatum* culture, regeneration frequency was 54% in control population. Similar to *D. mirbellianum,* adverse effect of ion-beam irradiation was observed on PLBs irradiated at doses from 2 Gy onwards. In general, the regeneration frequency was inversely proportional with irradiation doses. However, unlike *D.mirbellianum*, regenerated shoots were observed in 34% and 22% *D. crumenatum* PLBs irradiated at doses of 6 and 8 Gy, respectively, suggesting *D.crumenatum* was slightly more resistant to radiation than *D. mirbellianum.*

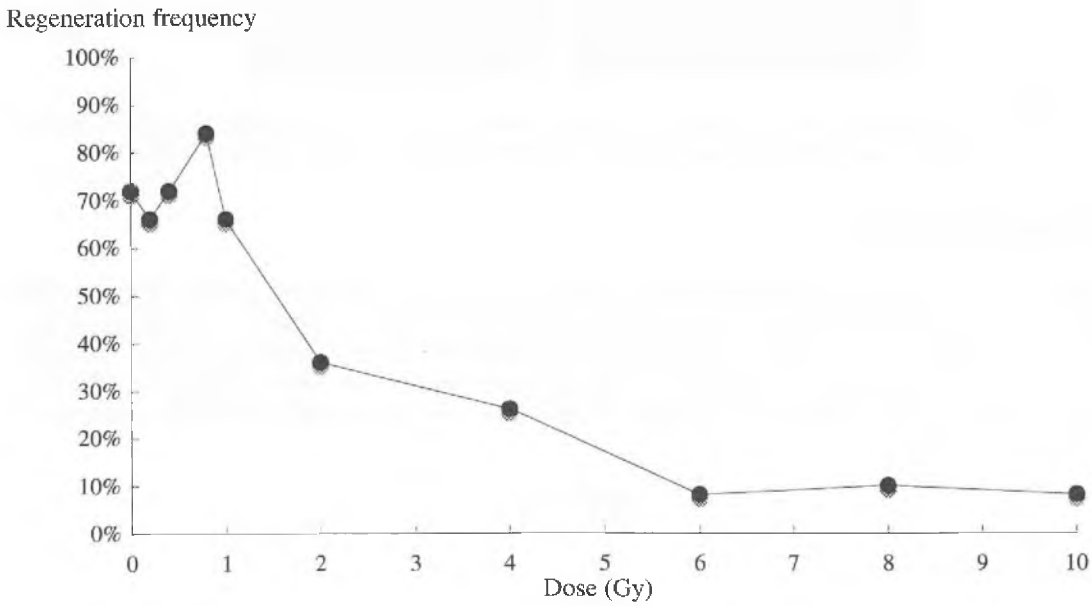

Figure 5. The dose response curve of shoot regeneration in *D.mirbellianum* PLBs irradiated by ion beams.

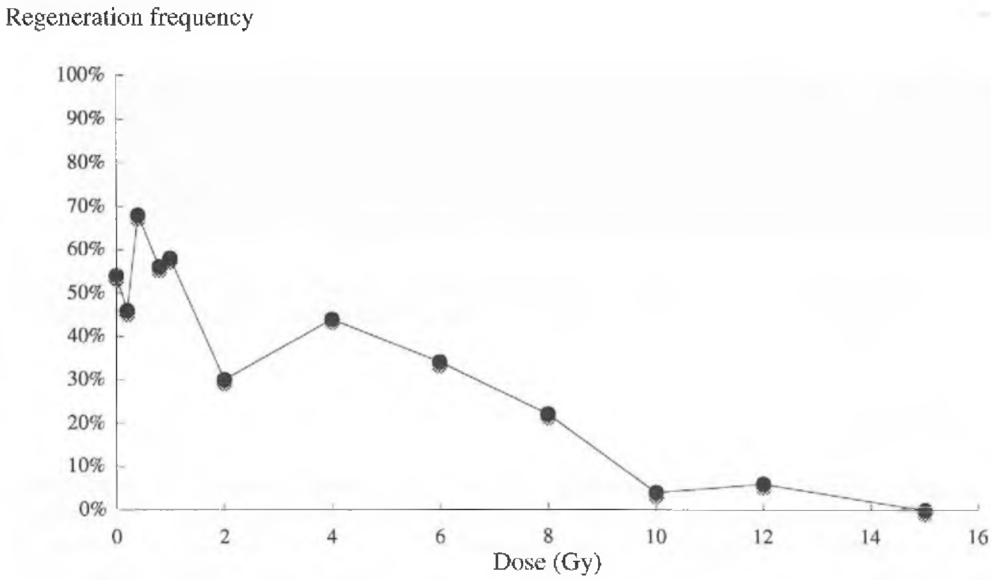

Figure 6. The dose response curve of shoot regeneration in *D.crumenatum* PLBs irradiated by ion beams.

***In vitro* observation**

i) *Dendrobium mirbellianum*

Mutation effects of carbon ions could be observed at the tissue culture stage on some irradiated cultures. In *D. mirbellianum*, chlorophyll mutation (variegated light yellow-green leaves) was detected in one of the cultures irradiated at 0.4 Gy (Figure 7). These cultures were left to grow into complete rooted plantlets in order to increase the chance of survival during hardening process. However, they could not survive the glasshouse condition and died after two weeks of transfer.

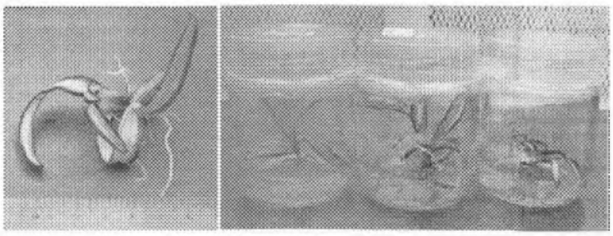

Figure 7. Chlorophyll mutation observed in 0.4-Gy irradiated *D.mirbellianum* culture.

i) *Dendrobium crumenatum*

Leaf morphological variations were also observed in a number of *D. crumenatum* cultures. Characteristics of leaf mutants are shown in Figure 8 (a-d), while the control is shown in Figure 10e. Figure 10a, 10b and 10c show shoot clumps regenerated from PLBs irradiated at 2 Gy, while Figure 10d shows shoot clumps irradiated at 0.2 Gy. In Figure 10a, variations could be observed in the shape of the leaves whilst in Figure 10b, in the elongation of shoot stem. Majority of the cultures irradiated at 2 Gy demonstrated the same leaf pattern as in Figure 10c. Another radiation effect (slow growth) was observed in a small number of cultures irradiated at 0.2 Gy. The variations were not found in the control populations. Therefore, these could be considered as potential mutants caused by radiation and not somatic variations caused by tissue culture effect.

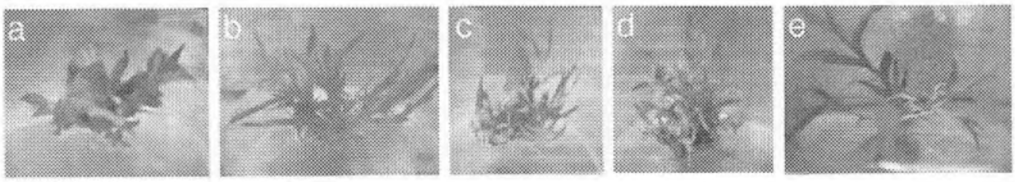

Figure 8. Regenerated shoots that show some abnormalities compare to the control (e). Shoots were regenerated from cultures of *D. crumenatum* irradiated by 2 Gy (a, b, and c) or 0.2 Gy (d) ion beams.

Observation in glasshouse

In glasshouse, plantlets of *D. mirbellianum* were found to grow slower than plantlets of *D. crumentum*. Unlike irradiated *D. mirbellianum*, some *D. crumenatum* plants have already been flowering. The length of blooming period was recorded. No extension of blooming period was observed in irradiated plantlets as compared to the controls, which bloom for only one day. Details on morphological changes observed in flowering mutant plants are given in Table 1, whilst variations on the flower shapes and sizes are shown in Figure 9. One of the 6.0-Gy irradiated plantlets shows an increase in flower width. The flower measures 55 mm across compare to that of the control, which was approximately 49 mm. A plant in 0.2-Gy population exhibited a longer flower stalk which measures 31.2 cm compare to that of the control which is about 15 cm length. The change was shown in Figure 10.

Table 1. Morphological mutation of flowering *D.crumenatum* plants irradiated by ion beams.

Dose (Gy)	Number of flowering plants	Number of mutants						total	% Mutant
		Flower morphology					Plant form		
		Large flower	Small flower	Long stalk	Different shape	Different orientation	Dwarf		
0	32							0	0
0.2	7		1	1				2	28.57
0.4	6							0	0
4.0	19					1		1	5.26
6.0	2				1			1	50.0
8.0	23		1					1	4.35
10.0	10	2						2	20.0
20.0	7						7	7	100.0

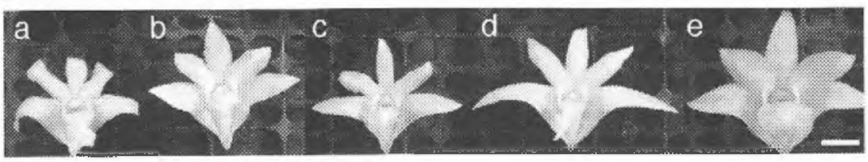

Figure 9. Flowers of *D. crumenatum* regenerated from PLBs irradiated at 0 (a; control) 0.2 (b), 4.0 (c), 6.0 (d), and 8.0 (e) Gy. The bar indicates 10 mm. Flower width (mm) was 49.0 (a), 44.5 (b), 37.2 (c), 55.0 (d), and 48.0 (e).

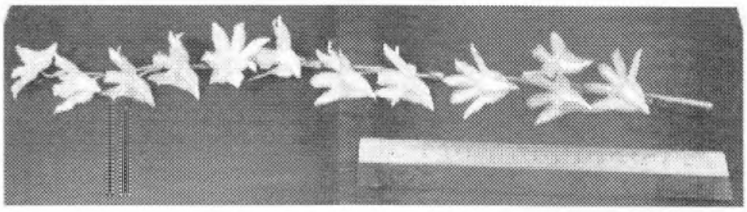

Figure 10. A mutant with longer flower stalk was observed in the regenerated plant irradiated at 0.2 Gy.

CONCLUSION

Inhibitory effect of ion-beam irradiation on shoot regeneration was observed on both *D. crumenatum* and *D. mirbellianum* PLBs irradiated at doses of 2 Gy and above. Glasshouse observations showed that irradiated plantlets of *D. mirbellianum* grew slower than irradiated *D. crumentum* plantlets. A number of potential *D. crumenatum* mutants with different flower morphology and size were successfully generated. However, genetic inheritability if these mutations need to be confirmed in next generation. At present, screening of the irradiated plant population are still on-going. More data on morphological mutations will be collected as the plants flowering. In addition, more data are also needed to confirm the relationship among irradiation dose, regeneration frequency and mutation effects.

ACKNOWLEDGEMENTS

This project was carried out under the Bilateral Research Cooperation between JAEA and Nuclear Malaysia.

REFERENCES

FAMA (2000) http://www.agrolink.moa.my

Guus Wijchman (2005). Malaysia Orchid. FlowerTECH 2005. 8(5):6-8.

Hase Y, Yamaguchi M, Inoue M, Tanaka A (2002) Reduction of survival and induction of chromosome aberrations in tobacco irradiated by carbon ions with different LETs. International Journal of Radiation Biology, **78:** 799-806

Murashige, T. and Skoog, F. (1962) A revised medium for rapidly growth and bioassay with tobacco tissue culture. Physiol. Plant. 15: 473-497.

Mohd. Khairol, M.A., Noor Auni, H. (1991) Potensi dan kajian kemungkinan penanaman orkid secara komersial. Published by MARDI (Malaysian Agricultural Research and Development Institute), P.O. Box 12301, 59774 Kuala Lumpur, Malaysia.

Mohd. Nazir, B., Sakinah, A. (2001) Molecular techniques as complementary tools in orchid mutagenesis. *Proceedings of the 2001 FNCA Workshop on Agriculture: Plant Mutation Breeding & Biofertilizer, August 20-24, Bangkok, Thailand,* JAERI, 91-102.

Mohd Nazir, B., Sakinah, A., Affrida, A.H., Zaiton, A., A. Tanaka, N. Shikazono, Y.Oono and Y. Hase (2003) Mutation Induction in Orchids using Ion Beam. TIARA Annual REPORT 2003, JAERI-Review 2004-025 45-47.

Nagatomi, S., Tanaka, A., Kato, A., Watanabe, H., Tano, S. (1996) Mutation induction on chrysanthemum plants regenerated from *in vitro* cultured explants irradiated with C ion beam. *TIARA annual report 5*, 50-2.

Okamura, M., Yasuno, N., Ohtsuka, M., Tanaka, A., Shikazono, N., Hase, Y. (2003) Wide variety of flower-color and -shape mutants regenerated from leaf cultures irradiated with ion beams. Nucl. Instrum. Methods Physic. Res. B 206: 574-578.

Rahman, A., Nakasone, A., Chhun, T., Ooura, C., Biswas, K.K., Uchimiya, H., Tsurumi, S., Baskin, T.I., Tanaka, A., Oono, Y. (2006) A small acidic protein 1 (SMAP1) mediates responses of the Arabidopsis root to the synthetic auxin 2,4-dichlorophenoxyacetic acid. *Plant J. 47*, 788-801.

Sakinah, A. and Mohd Nazir, B. (2002) Increasing characteristic variations in Dendrobium orchid through acute irradiation. 17th World Orchid Conference and Show, Shah Alam, Malaysia, April 26-May 2, 2002.

Shikazono, N., Yokota, Y., Kitamura, S., Suzuki, C., Watanabe, H., Tano, S., Tanaka, A. (2003) Mutation rate and novel *tt* mutants of *Arabidopsis thaliana* induced by carbon ions. *Genetics.163*, 1449-55