THESIS APPROVAL

GRADUATE SCHOOL, KASETSART UNIVERSITY

Master of Science (Tropical Agriculture)

DEGREE

Tropical Agriculture — **FIELD**

Interdisciplinary Graduate Program — **PROGRAM**

TITLE: Effects of Meta-topolin and Gamma Irradiation on Pineapple under Three *In Vitro* Systems

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THESIS

EFFECTS OF META-TOPOLIN AND GAMMA IRRADIATION ON PINEAPPLE UNDER THREE *IN VITRO* SYSTEMS

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A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Master of Science (Tropical Agriculture)
Graduate School, Kasetsart University
2010

Tesfay Tekelhymanot 2010: Effects of Meta-topolin and Gamma Irradiation on Pineapple under Three *In Vitro* Systems. Master of Science (Tropical Agriculture), Major Field: Tropical Agriculture, Interdisciplinary Graduate Program. Thesis Advisor: Associate Professor Surawit Wannakrairoj, Ph.D. 66 pages.

A proper dose of multiplication-inducing plant growth regulators, an appropriate culture method and an appropriate dose of mutagen are of primary importance for *in vitro* mutation induction. The present investigation assessed effects of meta-topolin (mT), a new promising cytokinin for micropropagation, and gamma irradiation on shoots of *Ananas comosus* 'Patawia' in shaking liquid media (with 13 µM µmol. $m^{-2}.s^{-1}$ PPF), solid media (with 26 µM µmol. $m^{-2}.s^{-1}$ PPF) and non-airflow temporary immersion system (TIS) (with 8 µmol. $m^{-2}.s^{-1}$ PPF). In each culture system, 2.5 or 5 µM mT with or without 2.5 µM NAA were compared with the MS basal medium for the shoot proliferation rate. After six weeks, the liquid medium with 2.5 µM mT led to the highest shoot multiplication rate of 15.75 plantlets per half-shoot explant in shaking system. Whilst the one with 5 µM mT was best for solid medium, with 6.75 plantlets per explant. The hourly immersion time of 4 minutes for TIS gave similar result to that of 6 minutes. However, the hourly immersion time of 4 minute with 2.5 or 5 µM mT containing media yielded the highest shoot proliferation rate for TIS. When the best culture condition of the three systems were compared for six weeks, it was revealed that the shaking liquid medium yielded the highest multiplication rate of 11.96 folds with the best growth. Six-weeks old plantlets from the three *in vitro* systems were subjected to acute gamma irradiation of 0, 40, 50, 60 and 70 Gray (Gy) at the rate of 5.58 Gy per minute. After six weeks, the 50% growth reduction dose (GRD_{50}) was determined to be approximately at 51.52 and 63.8 Gy for TIS and shaking liquid culture, respectively. However, GRD_{50} for the solid media was outside in the tested range.

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ACKNOWLEDGEMENTS

I would like to extend my sincere appreciation and gratitude to my advisor, Assoc. Prof. Dr. Surawit Wannakrairoj for his valuable academic guidance and enthusiasm shown throughout this study. His editorial comments and suggestions were also particularly significance throughout the preparation of this manuscript. I would also like to express my profound acknowledges to my thesis advisory committee members, Norongchai Pipattanawong (Dr.), for his valuable comments, and suggestions for my thesis manuscript and encouragement throughout my study.

The financial assistance from Rural Capacity Building Projects of under Ministry of Agriculture is sincerely acknowledged.

I am indebted to Assoc. Prof. Dr. Rungsarid Kaveeta for his technical comments regarding experimental designs and statistical analyses. The kind assistances and encouragements of all professors in my department, as well as my Thai and Ethiopian friends were also of special significance in minimizing my burdens in the course of the study. My special gratitude and thanks also go to Beyene Dimtsu, Surafel and Tekle and all others Ethiopia friends for their consistent support and encouragement all along my study periods enduring all the burdens of loneliness during the study.

Last but not least, I would like to express my gratitude to my family, whose consistent encouragements and motivations were the driving forces that helped me confront all the challenges. I am profoundly grateful to my mom and dad for their consistent encouragement all along my life that inspired me to attain this goal. My love, brothers and sister do also deserve a warm and sincere acknowledgement for their love and care.

Tesfay Teklehaymanot

April, 2010

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BA	N^6- benzyladenine or N^6 - benzyleaminopurine
2, 4-D	2, 4 – dichlorophenoxy acetic acid
DNA	Deoxyribonucleic acid
DW	Dry weight
DMRT	Duncan's Multiple Range Test
FW	Fresh weight
g	Gram
Gy	Gray
Hr	Hour
2iP	6 - (2- dimethylallylamino) purine
M	Molar
m	Meter
mM	Millimolar
min	Minute
ml	Millilitre
MS	Murashige and Skoog medium
N	Normal
NAA	1 - naphtalene acetic acid
PIB	Periodical Immersion Bioreactor
PPF	Photosynthetic Photon Flux
rpm	Rotation per minute
s	Second
TIS	Temporary Immersion System
µg	Microgram
µl	Microlitre

EFFECTS OF META-TOPOLIN AND GAMMA IRRADIATION ON PINEAPPLE UNDER THREE *IN VITRO* SYSTEMS

INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) is the third most important tropical fruits crops after banana and mango (Kole, 2007; Yaacob *et al.*, 1995). Its fruit is consumed predominantly as fresh or canned fruit. The total world production of pineapple was about 20.7 million tons in 2008 and approximately 90 % is consumed in the country it is produced (FAO, 2009). The world top pineapple producer included Thailand, Brazil and the Philippines with total production of 2.705, 2.561 and 1.834 million metric tons, respectively (USDA, 2006). The world pineapple trade consists mainly of processed products; i.e. canned slices, chilled fresh cut chunks and spears, juice and juice concentrates (Rohrbach *et al*. 2003).

Pineapple is also the only source of bromelain, one of the protease enzymes used in pharmaceutical products and as a meat-tenderizing agent. Moreover, the stems and leaves of pineapple are also used as sources of fiber (Anonymous, 2003). Pineapple fiber has been processed into paper with remarkable qualities of thinness, smoothness and pliability (Chattopadhyay and Sadhu, 2001). Processing wastes in the form of shell, core materials and centrifuged solids from juice production are used as animal feed (Anonymous, 2003). In another word, all parts of the plant are significantly useful.

In spite of all the above facts on the use of pineapple, the pineapple industry depends on a few cultivars (mainly smooth cayenne) making it extremely vulnerable to the threats of pests and diseases in different part of the world (Bartholomen *et al*, 2003; Botella and Fairbairn, 2005). Although smooth cayenne has been the backbone of the global pineapple industry for more than a century, currently its supply declined resulting in an increase price of pineapple (FAO, 2009). The cultivar is susceptible to heart rot and root rot diseases. A disease resistance is thus sought after (Suneerat, 2009). The producers have also complained that smooth cayenne had declined in

vigor and yield over many years of replanting. This is an indicator of the urgency to develop suitable varieties. However, cultivar improvement with classical breeding approaches like hybridizations and selection has been very little in progress when compared to other crops due to nature of the crops like self incompatibility and heterozygousity as well as long juvenile period (Botella and Fairbairn, 2005).

In vitro mutation induction is an alternative way for cultivar improvement (Ahloowalia, 1997). The combination of *in vitro* culture and mutation induction techniques provides highly efficient method to improve horticultural crops (Predieri, 2001). Tissue culture allows handling of large populations for mutagenic treatment and rapidly cloning of selected variants. It also offers the possibility to isolate a solid mutant from a chimeric mutant (Predieri, 2001). In pineapple, spineless and chlorophyll mutants that look like "ornamental bromeliads" were developed via *in vitro* regeneration of irradiated explants in the Philippines (Lapade *et al*., 2002). Appropriate protocol for an effective micropropagation is desirable for an *in vitro* mutagenesis of pineapple (Van Harten, 1998). A new aromatic cytokinin, meta-topolin (mT) has been reported to be more effective than BA, common cytokinin used for pineapple micropropagation (Bairu *et al*., 2007; Dobranszkil *et al*., 2005; Escalona *et al*., 2003; Werbrouck *et al*., 1995). However, there has been not work using mT for pineapple micropropagation.

Therefore, it is interesting to explore potential use of mT for shoot multiplication as well as to determine the radiosensitivity of pineapple under three commonly used *in vitro* culture systems; namely solid media, shaking liquid media and temporary immersion system (TIS). The information from the study will contribute to the success of *in vitro* mutagenesis in pineapple.

OBJECTIVES

1. To determine the best concentration of meta-topoline and NAA for multiplication of pineapple in three *in vitro* systems.

2. To assess the radiosensitivity of pineapple under three *in vitro* systems with meta-topolin.

LITERATURE REVIEW

Botanical Characteristics

The family Bromeliaceae comprises of 56 genera with 2,921 species, classified into three subfamilies: Pitcarnioideae, Tillandsioideae and Bromeliadeae (Luther and Holst, 2004). Bromeliaceae is set apart from other monocots by the unique scale like multicellular hairs and the unusual conduplicate (folded lengthwise) and spiral stigmas (Gilmartin and Brown, 1987). The subfamily Bromeliadeae shows a tendency towards the fusion of floral parts. *Ananas* is the only genus whose flowers and bracts are completely merged into a single sorose-type parthenocarpic fruit (Bartholomew *et al*., 2003). Pineapple (*Ananas comosus* (L.) Merr.) has spiny or smooth leaves (Luther and Sieff, 1998).

Pineapple is a perennial monocarpic herb. Each shoot flowers only once and dies. After fruiting, the side shoot takes over. The flowers, 100-200 in number, are hermaphrodite. Each flower is at the axils of a bract. Daily, about 5-10 flowers open from the base upward (acropetal succession) for 10-20 days. However, some cultivars flower in a very disorder manner. The flowers are self-incompatible. (Chattopadhyay and Sadhu, 2001). Anthesis normally takes place within a day after the flower is opened. Soon after anthesis, stylar canals are closed by a mucilaginous plug. One or two weeks later by cellular occlusion (Bartholomew *et al.*, 2003). In the mature fruit, the stylar canals are completely closed. The edible parts of the fruit consist chiefly of the ovaries, the bases of sepals and bracts, and the cortex of the axis (Ray, 2002). The seeds are approximately 3–5 mm long and 1–2 mm wide, flat on one side and curved on the other, with a pointed end. They contain a hard flinty endosperm and a minute embryo enclosed in extremely tough and leathery, brown to black coat, roughened by numerous longitudinal ridges (Bartholomew *et al.*, 2003).

The pineapple cultivars are classified in five groups: Cayenne, Queen, Spanish, Brazilian, and Maipure. A subgroup of Cayenne group, smooth cayenne, has fruit of high quality and cylindrical shape ideal for canning. For a smooth cayenne

subgroup, the leaves have spines which confined to the tips. Its fruit ripens progressively, turning yellow from the base to the top and form dense rosette with wide blades (Bartholomew *et al*., 2003).

Ecology and Distribution

Pineapple is native to South America. The centre of origin is thought to be in the northern Brazil, Colombia, Venezuela and northern Argentina. These regions contain the greatest level of diversity within the species (Bartholomew *et al*., 2003).

Pineapple is a true xerophytic crop. It can store water in specialized storage cells of the leaves, thus can tolerate drought to a great extent. The useful feature of pineapple is its adaptation to areas of low rainfall, although productivity is reduced in drought conditions. Moreover, pineapple, as most Bromeliaceae, has the crassulacean acid metabolism (CAM) photosynthetic pathway (Bartholomew *et al.*, 2003). Production at the more extreme latitudes is mainly restricted to areas where the climate is moderated by the ocean. The crop cannot tolerate freezing temperatures. The optimum day/night temperature for growth is about 30/20 ^{o}C (Chattopadhyay and Sadhu, 2001).

The light saturation point of pineapple plants is at a photosynthetic photon flux (PPF) of about 500 µmol m^{-2} s^{-1}. However, it is probable that the degree of irradiance can alter the pattern of CO_2 fixation. The intensity of CAM tends to decrease as irradiance increases, at least until saturating levels are attained. The optimum day length is from 10 to 16 hours (Nose *et al.*, 1986).

Plant Tissue Culture Systems

The rate at which cultures grow and produce shoots during micropropagation can be influenced by the physical nature of the medium (George and Davies, 2008). The growth response of plants to solid and liquid media varied among genera or species or even cultivars (Takayama and Akita, 2006). Miller and Murashige (1976) demonstrated that selection between agar-gelled solid media and liquid media

(whether shaken or stationary) should not be undertaken arbitrarily. In their experiments, cordyline explants survived equally well on solid and liquid medium. However, none of the scindapsus explants stayed alive on a rotated liquid medium. Only a small proportion survived on a static liquid medium, while nearly all grew satisfactorily on a filter paper support or on the solid medium.

The advantages of using solid medium are that the explants are ensured to suitable anchor and stay at the same orientation throughout the culture. The plant material has a great contact area with air. On the other hand, its disadvantages are that rate of growth and multiplication are slow and toxic exudates from explants do not diffuse away (George and Davies, 2008). As a result, poor survival rates during acclimatization was obtained (Escalona *et al.*, 1999; Escalona *et al.*, 2007). Another disadvantage is that the use of solid media increases the time taken to clean containers and glassware for re-use. These factors make the use of solid media costly.

The use of liquid medium facilitates nutrient uptake due to its complete contact with the explants (Gupta *et al.*, 1981). Multiplication rate of pineapple in liquid medium was significantly higher than in solidified medium (Almeida *et al*., 2002). In addition, toxic metabolites, which may accumulate in the vicinity of the tissues are effectively dispersed. Jackson *et al.* (2003) stated that there may also be direct effects of low oxygen tensions on membrane functioning, on water and ion uptake capacity. One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots are perpetually submerged and may have hyperhydric reaction which will have a negative effect on survival rate after deflasking. Santamaria *et al.* (2000) reported that without obvious hyperhydricity, micropropagated plantlets could show lesions in growth and development. These can adversely affect the survival rate after plantlets are transferred to the acclimatization. Davies and Santamaria (2000) stated that the problem can be attributed to low oxygen tensions in media as well as accumulation of other gases in the culture vessels. Sharp (2002) also reported that ethylene production enhanced at low oxygen tension can dramatically restrict shoot growth and function.

Temporary immersion system (TIS) was developed to combine the advantages of solid and liquid medium and environment condition. It provided intermittent availability of medium and allowed air contact of the growing plants (McAlister *et al.*, 2005). Plantlets in TIS assimilated significantly more nutrients than those in solid medium. The photosynthetic capacity induced by TIS resulted in consistent increase of sugar and nitrogen uptake as well as dry weight and leaf area of the plantlets (Escalona *et al.*, 2003).

Plantlets in TIS had enhanced physiological behavior showing more growth in both *in vitro* and *extra vitrum* than plantlets on solid media culture. Plantlets in TIS uptake more oxygen during elongation phase and less during multiplication phase than solid media (Escalona *et al.*, 2007). Moreover, plants from TIS produced thicker leaf chlorenchyma and aquiferous parenchyma, lower stomata density and more epicuticular wax than those on solid medium. During their acclimatization, plants from TIS had also substantially higher photosynthetic rates than those from a solid medium. Plants from TIS had subsequent better performance in acclimatization (Yang and Yeh, 2008). However, Damiano *et al.* (2005) reported that the multiplication rate of *Prunus* species and a *Malus* rootstock in the TIS did not differ from solid media after 60 days.

Wawrosch *et al.* (2005) compared the performance of *Charybdis numidica* in solid, liquid and temporary immersion system. They observed higher regeneration rate without hyperhydration of the shoots on solid than liquid and temporary immersion system. Damiano *et al.* (2005) reported that the multiplication rate and growth of *Prunus* and a *Malus* rootstock in temporary immersion system with 30 or 60 minutes daily immersion was the same as on solid media. Stationary liquid culture resulted in reducing multiplication rate, chlorophyll and fructose contents. It also induced hyperhydricity and necrosis. On the other hand, Escalona *et al.* (1999) reported that pineapple had the highest shoot multiplication rate of in twin flask temporary immersion system respectively as comparing to solid (4 folds) and shaking liquid media (3 folds).

Important factors for temporary immersion system

For most of temporary immersion systems types, the volume of the container is much greater than those of conventional procedures (Krueger *et al*., 1991). As head space is an important factor that affects multiplication rate as well as the quality and quantity of plantlets from temporary immersion systems (Roels *et al*., 2005). Lai *et al*. (1998) and Zobayed *et al*. (1999) stipulated that the high multiplication rates, increases in fresh weight and provide good quality of plantlet under temporary immersion system may not only be due to the uptake of nutrients and hormones over the whole plant surface, but also the daily multiple air exchange.

Volume of the liquid medium is particularly important to optimize the liquid medium volume when using temporary immersion systems without medium renewal, such as the twin flasks and RITA® systems or tilting and rocker machines. Lorenzo *et al.* (1998) found an optimum volume of medium per explant for sugarcane shoot proliferation in the BIT® twin flasks system. An increase in multiplication rate from 8.3 shoots per 30 days to 23.9 shoots per 30 days was obtained by increasing the volume of medium from 5 to 50 ml per explant. However, the volume of medium used did not affect the length of the shoots formed. Higher volume than 50 ml per explant proved to be less efficient. Using the same temporary immersion system, Escalona *et al*. (1999) demonstrated that an optimum medium volume for pineapple shoot proliferation was 200 ml per explant. In this case, higher volume than 200 ml per explant also led to a drop in the proliferation rate.

Immersion time intervals in temporary immersion system plays a decisive role in influencing the multiplication rates as these factors affect nutrient supply and composition of the internal atmosphere in the culture vessel (Jimenez *et al*., 1999). Jackson (2005) stated that an aqueous cover interferes strongly with gas exchange to the outer tissue or cell surface since gas diffusion rates are approximately 10,000 times slower in water than in air. This impact is increased with the depth of the aqueous cover complete submersion, or submersion of the plants too frequently for long periods and reduce gaseous exchange for photosynthesis and respiration. Hence,

optimum immersion frequencies must be determined to ensure sufficient gas exchange to support growth and development (Hempfling and Preil, 2005).

The immersion times used for different work vary considerably due to the variety of species, micropropagation processes and temporary immersion systems used. Long daily immersion times (1 h every 6 h) prove to be efficient for potato tuberization (Teisson and Alvard, 1999). Whereas, short immersion time (2 min every 3 hr) stimulate high multiplication rate in pineapple (Escalona *et al*., 1999). Likewise, very frequent immersions (30 sec every 30 sec) can prove to be highly efficient in tilting machines for grapevine shoot propagation (Harris and Mason, 1983).

Meta-topolin in Tissue Culture

Presently, it is widely accepted that derivatives of BA, such as the topolins are as naturally occurring cytokinins. Meta-topolin (mT) was first isolated from mature poplar leaves and identified as (N^6-(3 - hydroxybenzylamino) purine). It has been used in tissue culture of some plant species as cytokinin.

Reports indicate that aromatic cytokinins have antisenescence effects and help stability of micropropagation than the commonly used benzylaminopurine (BA) (Bogaert *et al.*, 2006; Holub *et al.*, 1998; Tarkowska *et al*., 2003). Baroja-Fernandez *et al*. (2002) reported that the potato cultivar 'Kennebec' with high aromatic cytokinin content (92%) showed high *in vitro* growth and survival. On the contrary, other potato cultivar 'Jaerla' with relatively high level of isoprenoid types cytokinins had low viability. Addition of *meta*-topolin to the culture medium showed a significant improvement of the viability of the latter cultivar.

The activity of mT was also comparable to that of zeatin (the most active isoprenoid cytokinin) in the bioassays (Strnad *et al.*, 1997; Tarkowska *et al*., 2003). It is also less toxic and easier degradable than BA in *Spathiphyllum floribundum* (Bairu *et al.*, 2008). Furthermore, Werbrouck *et al*. (1995) report that mT metabolite, O-glucoside, was degraded easier than metabolite of BA. *Spathiphylum flouribundum*

treated with 10 µM mT produced a significantly higher number and longer roots than BA during rooting and acclimatization. Moreover, plantain multiplication was also better in mT (12.5 folds) than BA (8.5 folds) at the same concentration (4.4 µM) after 28 days (Escalona *et al.*, 2005).

The conditioning of apple shoots for three weeks on a medium supplemented with 2.07 or 6.21 µM mT yielded a significant decrease in the rate of hyperhydric shoots while significantly increased the number of regenerated shoots per leaf segment (Dobranszkil *et al.*, 2005). Bairu *et al.* (2007) also showed that mT was more effective than BA on controlled hyperhydricity and multiplication in *Aloe polyphylla*.

Micropropagation of Pineapple

Pineapple can be propagated vegetatively or sexually. However, seeds have low viability hence are not a preferred means of propagation. Pineapple is propagated vegetatively through crowns, slips, suckes and ratoon suckers (Chattopadhyay and Sadhu, 2001; Bartholomew *et al.*, 2003). However, micropropagation of pineapple has many advantages over conventional methods of vegetative propagation, in part due to its efficient and rapid increase of selected varieties (Drew, 1990; Kiss *et al.*, 1995 and Mathews *et al.*, 1979). In addition to yielding abundant planting material of uniform age, micropropagated plants have been known to mature and fruit all at one time. The use of micropropagated pineapple plants as planting material can help to minimize the problem of “natural flowering” which is sporadic and rampant in pineapple cultivation growing from other vegetatively parts (Mhatre, 2007).

Smith and Drew (1990) have reviewed many of the applications of tissue culture for plant propagation and improvement. Applications for pineapple propagation and improvement include: micropropagation via axillary-bud proliferation, adventitious-bud proliferation and regeneration from callus cultures, and culture of excised protoplasts, ovules or anthers.

Plant growth regulators for pineapple micropropagation

Plant hormones are naturally produced substances that regulate plant growth and development. A large number of related chemical compounds synthesized artificially are also used to regulate the growth of cultivated and *in vitro* grown plants. These man-made compounds are called plant growth regulators (Ska *et al*., 2001). Different plant growth regulators show varied degrees of activities in affecting shoot formation *in vitro* depending on their concentration and types (Mok *et al*., 1987).

Reports on supplemented plant growth regulator types and their interaction effects on *in vitro* shoot multiplication of pineapple are varied. Application of kinetin or BA without auxin was sometime suggested for pineapple multiplication. Pineapple was well proliferated on an MS solid medium supplemented with 8.8 - 31 µM BA (Almeida *et al.*, 1995; Bhatia and Ashwath, 2002; Danso *et al.*, 2008). On the other hand, an MS liquid medium supplemented with 3.55–22 µM of BA was recommended for shoot proliferation (Be and Debergh, 2006; Danso *et al.*, 2008). For kinetinin, Fotso *et al*. (2001) reported that pineapple shoot induction and proliferation was obtained in an MS liquid medium supplement with 27.88 µM kinetin.

Combination of BA with auxin was sometimes shown to be the best for *in vitro* multiplication of pineapple. Abdelhamid *et al.*, (2008) found out that combined application of 14.65 µM BA and 10.28 µM IAA could induce high pineapple shoot multiplication (19 fold in 75 days). Similarly, Dal Vesco *et al*. (2001) showed that an MS medium supplemented with 4 µM BA and 2 µM NAA yielded a high shoot multiplication rate from axilary bud (13.5 fold in 45 days). Axillary buds were also multiplied at 4 to 9 folds in 35 to 42 days on an MS medium supplemented with 8.87 µM BA and 2.26 µM 2.4-D (Sripaoraya *et al.*, 2003). On the other hand, Barboza *et al*. (2004) reported that 8.88 µM BA and 10.74 µM NAA was best for shoot proliferation of a smooth cayenne cultivar whereas 8.88 µM BA was best for proliferation of its hybrid. This indicated genotype specific response to plant growth regulator on shoot proliferation.

Kinetin was also reported to be effective in combination with auxin. Dolgov *et al.* (1998) found that the best regeneration could be achieved in 46 days when the leaf explants or callus were placed on media containing (2iP + kinetin) to (NAA + IBA) ratio of 2:1 or 1:5. In addition, 1 mg L^{-1} 2iP, 1 mg L^{-1} kinetin, 5 mg L^{-1} NAA and 5 mg L^{-1} IBA was gave high regeneration of plantlets from callus. On the other hand, axillary buds from crowns multiplied well (6 folds) with 9.29 µM kinetin, 9.67 µM NAA and 9.84 µM IBA on solidified MS medium (Soneji *et al*., 2002). Similarly, an average of 6 shoots per culture was obtained an MS medium supplemented with 10.74 µM NAA, 9.29 µM kinetin and 9.84 µM IBA (De Jarasu, 2004). Furthermore, 9.29 µM kinetin and 9.84 µM IBA plus casein hydrolysate were also suggested for pineapple micropropagation (Bordoloi and Sarma, 1993).

According to Huetteman and Preece (1993), addition of a second cytokinin to the proliferation medium could enhance shoot proliferation. Supporting this ideas, Kiss *et al.* (1995) suggested that using 25 µM kinetin and 20 µM BA could enhance shoot proliferation of pineapple.

When compared in equi-molar, zeatin was found to be more effective than kinetin or BA for shoot proliferation of smooth cayenne and queen pineapple (18 folds vs 14 folds vs 9 folds, respectively) (Fitchet, 1990). Similarly, Fotso *et al.* (2001) reported that higher level plantlet multiplication rate were obtained in MS liquid medium supplemented with 27.88 µM kinetin (11.9 fold) than that with 17.76 µM BA (6.7 fold) after 65 days.

In Vitro Mutation Induction

Mutations are defined as heritable changes in the DNA sequence that are not derived from genetic segregation or recombination (Van Harten, 1998). Variation can be randomly induced with mutagens treatment (Predieri, 2001). Mutagens have been successfully used in plant breeding programs to artificially generate genetic variation for the development of new varieties with the change of one or few characters improved traits such as increased yield, earliness, reduced plant height, and resistance

to disease (Maluszynski *et al.*, 2001; Nagatomi, 1996). Moreover, mutation induction is also a powerful tool for the investigation of gene function and expression (Kodym and Afza, 2004). It may also be the most effective way to inactivate an existing gene (Somsri *et a*l., 2008). Due to the variation caused by induced mutations is not essentially different from naturally spontaneous mutations, biosafety is not an issue of importance for a mutant (Heinze and Schmidt, 1995; Predieri, 2001).

Different mutation spectra are obtained with different types of mutagens which could be chemical or physical agents. Among the chemical mutagens the most commonly used mutagens are alkylating agent and azides. The example of alkylating agent are ethyl methane sulphonate (EMS), diethyl sulphate (DES) and methyl nitrous urea (Kodym and Afza, 2004). Physical mutagens include gamma rays, X rays, and ion beam. Ionization radiations have easy application, high mutation frequency and there is no residues risk. Ion beam has higher linear energy transfer to produce double-strand breaks than gamma- or X-rays (Tanaka, 1999). For a few decades, more than 2,252 mutant varieties have been officially released in the world (Maluszynski *et al*., 2001). A great majority of mutant varieties (64%) were developed by the use of gamma rays (Ahloowalia *et al.*, 2004).

Among the most important groups of traits induced by mutagenesis include plant size, blooming time and fruit ripening, fruit color, self-compatibility, self-thinning, and resistance to pathogens (Predieri, 2001). Induction of mutations offers the possibility to produce a limited number of desired genetic changes in genotypes and varieties, which are adapted to the local eco-climatic condition. However, this valuable technique is not yet fully exploited in fruit breeding.

Despite the importance of improved resistance against various diseases in fruit crops, only few successful examples of induced mutation are known. Mutant with higher level of resistance to black spot disease was obtained after gamma irradiation of ‘Gold Nijisseiki’ Japanese pear were reported (Anonymous, 1996). Saito *et al*. (2001) successfully screened mutants which were resistance to Alternaria blotch disease of apple (*Malus* × *domestica* Borkh.) from the irradiated shoots. In addition,

mutants which were resistance to bacterial heart rot disease was obtained after gamma irradiation of pineapple (Ibrahim *et al*., 2009).

Factors affect mutation induction by irradiation

The most important factor in irradiation of planting materials for mutation induction is the efficient dose. Kodym and Afza (2004) stated that before starting any mutation induction studies, it is most crucial to select suitable doses by testing two to three doses along with a control. Mutation can take place in any kinds of tissues but a single mutation event is restricted to one cell. Recovery of shoot apex after irradiation happens via regeneration either from the axillary buds or adventitious buds. The dose may alter the place and types of regeneration (Broertjes and Van Harten, 1978). A dose less than one kilo Gray is commonly used to induce mutation in seed materials, cutting or *in vitro* cultured plant materials (Ahloowalia and Maluszynski, 2001). In assessing the most appropriate gamma ray dose, determination of the $LD_{50,}$ the dose cause 50 % lethality, or GRD_{50}, the dose that cause 50 % growth reduction, must be conducted (Heinze and Schmidt, 1995; Liang *et al.*, 1996).

Correlations between the physiological status of plants and their radiosensitivity are often determined by the water content of the tissue. Exposure to gamma radiation causes hemolytic fission of hydrogen bond in the water molecules to give H and OH radicals. Hydroxyl radicals react with almost all molecules; e.g. protein , DNA and lipid in living cells (Meyers, 1995). Aseptic plant tissues are much more sensitive to radiation treatment than stem cuttings or seeds (Ahloowalia, 1997; Predieri, 2001). Moreover, different culture systems affect the water content in the explants (George and Davies, 2008). In addition oxygen could enhance biological radiation damage (Van Harten, 1998). In the study conducted by Roels *et al.* (2005), there was wide variation in the available of oxygen between the *in vitro* system in plantain. Solid medium had 15.1% while the temporary immersion system had 19.3% oxygen.

The condition under which the materials maintained before and after mutagenic treatments also affects the mutation frequency (Van Harten, 1998). Irradiation results in the competition between affected cells and unaffected cells (Patade and Suprasanna, 2008). Using the appropriate *in vitro* culture techniques could minimize the competition (Van Harten, 1998). As an outcome, a mutant cell has an equal chance to form shoot of a plant to a normal cell (Broertjes and Van Harten, 1978).

Mutation Induction in Pineapple

In pineapple, cayenne has been the most important group in commercial production for many years. It started as a single genotype but the occurrence of somatic mutation led to a number of distinguished clones within this group (Broertjes and Van Harten, 1978). According to an annual report of Japanese National Institute of Agrobiological Resource, pineapple mutant could effectively be induced by irradiation of leaf cutting and tissue culture (30 and 500 times, respectively more than spontaneous mutation from suckers) (Annonymous,1994).

Only few irradiation experiments on pineapple have been reported. Spineless and chlorophyll mutants that look like “ornamental bromeliads” were developed via regeneration of irradiated *in vitro* explants in the Philippines (Lapade *et al.*, 2002).

The LD_{50} of *in vitro* plantlets of pineapple was reported to 45 Gy in the shaking liquid media using 20 µM BA (Lokko and Amoatey, 1998). However, the LD_{50} of *in vitro* axillary bud of pineapple was observed to be 40 Gy on MS medium supplemented with 8.8 µM BA and 10.74 µM NAA (Lapade *et al*., 2002). HsuehShih and YuehShiah (2005) reported that increasing radiation dosage led to an increase in pineapple mutation rate.

MATERIALS AND METHODS

Planting materials: *In vitro* shoots of a smooth cayenne pineapple (*Ananas comosus,* 'Pattawia' kindly provided by Rajchamongkol University, Lumpang campus, were used as starting materials. They were grown and multiplied on a solid MS medium supplemented with 6.67 µM BA with six weeks interval of subculturing (Almeida *et al*., 2002).

Plantlets with 2-3 cm height were cut longitudinally. Each half was taken as experimental unit. The sections were placed on the media with cut surface downward. Three experiments were designed and executed as follow:

Experiment I. **Multiplication Rate of Pineapple Culture in Three *In Vitro* Systems with Different mT and NAA Concentrations**

The modified MS medium with 2.5 or 5 µM meta-topolin (N^6-(3-hydroxybenzylamino) purine) (Phyto Technology Laboratories™, USA) with or without NAA 2.5 µM were employed to determine their effectiveness on *in vitro* shoot multiplication of pineapple as compared to the basal media. The best combination of plant growth regulator for solid media, shaking liquid medium and temporary immersion system were separately investigated. The experiment was laid out in completely randomized design (CRD) with four replications.

For the solid media, the media were solidified with 6.5 gm L^{-1} agar-agar. Each 100 ml culture vessel contained 20 ml of culture medium. The culture vessels were placed under 26 µmol. $m^{-2}.s^{-1}$ Photosynthetic Photon Flux (PPF) from the daylight fluorescent lamps (TOSHIBA FL18W).

For shaking liquid culture, the explants were placed in 100 ml culture vessel containing 20 ml of culture media. The culture vessels were placed on 100 rpm orbital shaker under 13 µmol. $m^{-2}.s^{-1}$ PPF from the daylight fluorescent lamps.

For the temporary immersion systems (TIS), a non-airflow single flask on a rotary was used. The erlenmeyer vessels were put on the rotary wheel at the angle of 30 ° The rotary wheel had a clock wise rotation controlled by automated timer. A rough translucent polycarbonate sheet was folded in a 100 ml erlenmeyer flask to hold an explant above the media temporarily upon rotation (Fig. 1). In an immersion cycle, explants were hourly immersed for 4 or 6 minutes. Each vessel with an explant was used as an experimental unit and placed on rotary temporary immersion system. The 2x5 factorials of immersion times and plant growth regulator were employed. The culture vessels were placed on the non-airflow temporary immersion system under 8 µmol. $m^{-2}.s^{-1}$ PPF from the daylight fluorescent lamps.

The basal medium contained Murashige and Skoog (1962) basal media with 3% sucrose. The pH was adjusted to 5.8 with 1N potassium hydroxide or 1N nitric acid before autoclaving for 20 minutes at 121°C and 1.1 kg. cm^{-2}.

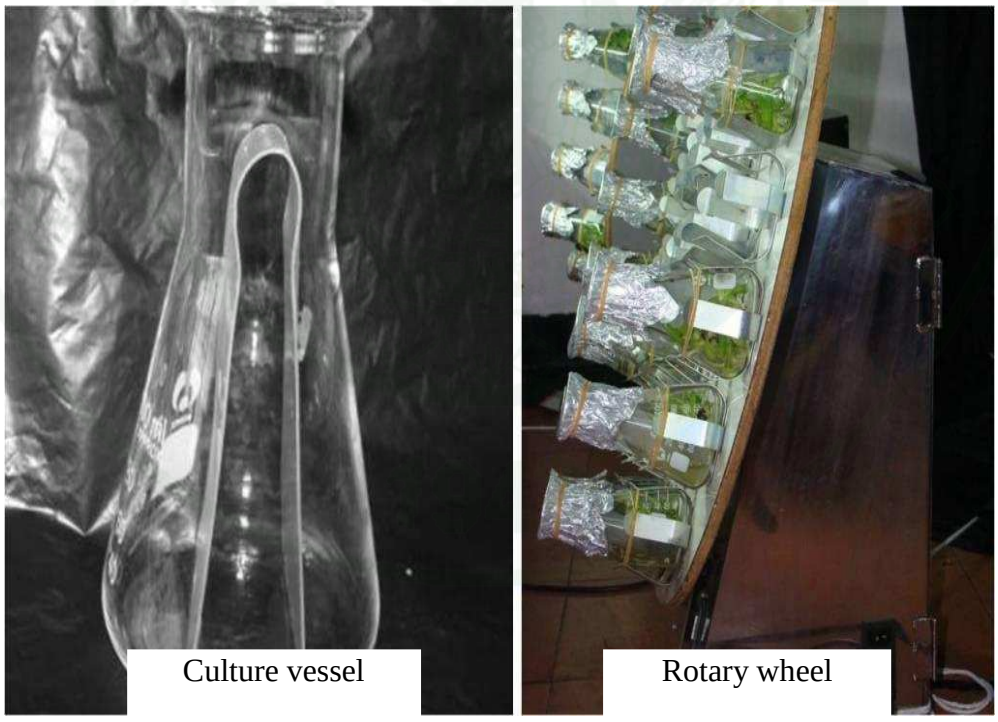

Figure 1 Non- airflow temporary immersion system for *in vitro* pineapple shoot multiplication.

Cultures were incubated at 27 ± 2°C under 16 hours photoperiod. After six weeks, data was recorded for the number of shoots per explant from each *in vitro* system and analyzed using the ANOVA, following by the Duncan's Multiple Range Test (DMRT).

Experiment II. **Comparison of Multiplication Rate of Pineapple among the Best Media from the Three *In Vitro* Systems**

The explants similar to those used in the experiment I was transferred to the best media for each *in vitro* system. The shoots were incubated under the same condition as in the experiment I and subculture every 6 weeks. The number of shoot per explant, height, fresh and dry weight were recorded and statistically compared. Dry weight was measured after drying the plantlets at 70°C for 6 days and repeated after 1 hour at the same temperature.

Experiment III. **Effects of Gamma Irradiation on Pineapple Shoots in the Three *In Vitro* Systems**

Uniform *in vitro* shoots of pineapple from the experiment II were multiplied on the best medium from the experiment I for the three *in vitro* systems. Four plantlets were placed in an empty sterilized bottle and subjected to acute gamma irradiation. Irradiation was done at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University, Bangkok, Thailand. Gamma rays of 0, 40, 50, 60 and 70 Gy in the rate of 5.58 Gy per minute were used. After irradiation, treated plantlets were promptly subcultured to the same media. The culture vessels were placed under the same condition as the experiment I.

A 3x5 factorials (3 *in vitro* system and 5 gamma rays dose) was employed in completely randomized design (CRD) with five replications. Total number of shoots per culture, height of the shoot and fresh and dry weight after 6 weeks were recorded. For the radiosensitivity test, 50 % growth reduction dose (GRD_{50}) was determined by

comparing the multiplication rate of the irradiated shoots and the non-irradiated ones in each *in vitro* system (solid, shaking liquid media and TIS).

Data analysis

All data were subjected to analysis of variance (ANOVA). Means were separated using Least Significance Difference (LSD) or Duncan's multiple ranges test (DMRT) as appropriate. The statistical analyses were carried out using IRRSTAT program (IRRI, 2005).

Place and Duration

The experiments were conducted in the Laboratory for Plant Cultivar Development, Department of Horticulture, Kasetsart University, Bangkok, Thailand from May, 2007- September, 2009.

RESULTS AND DISCUSSION

Experiment I. Multiplication Rate of Pineapple Cultured in Three *In Vitro* Systems with Different mT and NAA Concentrations

A. Shaking liquid media

In the shaking liquid media, most of the mT containing media led to higher shoot per explants than the basal one (Table 1). Among the media with only mT, shoot multiplication rate in the medium containing 5 µM mT was statistically the same as that in the control. The concentration might be supraoptimal for proliferation of pineapple shoots. Adding 2.5 µM NAA to mT containing media led to the multiplication rate enhancement only in the medium with 5 µM mT. However, it resulted in swollen and thicken leaves (Fig. 2). The morphological abnormality indicated hyperhydricity. The optimal mT concentration for multiplication was 2.5 µM resulting in 15.75 normal shoots per explant.

Hyperhydricity was common when BA was included in the media of *Aloe polyphylla* (Chukwujechwu *et al*. 2002). Bairu *et al*. (2007) reported that replacing BA with mT could control the problem of hyperhydricity in tissue culture of *A. polyphlla*. The result of the present experiment only agrees with the latter report when mT was used without auxin. Palni *et al.* (1988) reported that NAA might result in reduced natural cytokinin stability by enhancing cytokinin oxidase activities. Nordsrom *et al.* (2004) also indicated the negative effects of auxin on cytokinin. Similarly, NAA might be blinding activity of mT in controlling hyperhydericity in the present experiment.

The multiplication rate in the present experiment was lower than that of Danso *et al. (*2008). They obtained 29 folds increase with MD2 pineapple in 42 days using a. modified MS liquid medium with 22.2 µM BA and 10.74 µM NAA.

Table 1 Multiplication rate of 'Patawia' pineaple cultured in shaking liquid media with different mT and NAA concentrations for six weeks.

PGR in MS media (µM)		Multiplication (folds)
mT	**NAA**	
0	0	7.25c [1/]
2.5	0	15.75a
5	0	8.50c
2.5	2.5	13.00ab
5	2.5	11.50b
CV (%) 18.60		

[1/]Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test (DMRT) at P=0.05.

PGR= plant growth regulator.

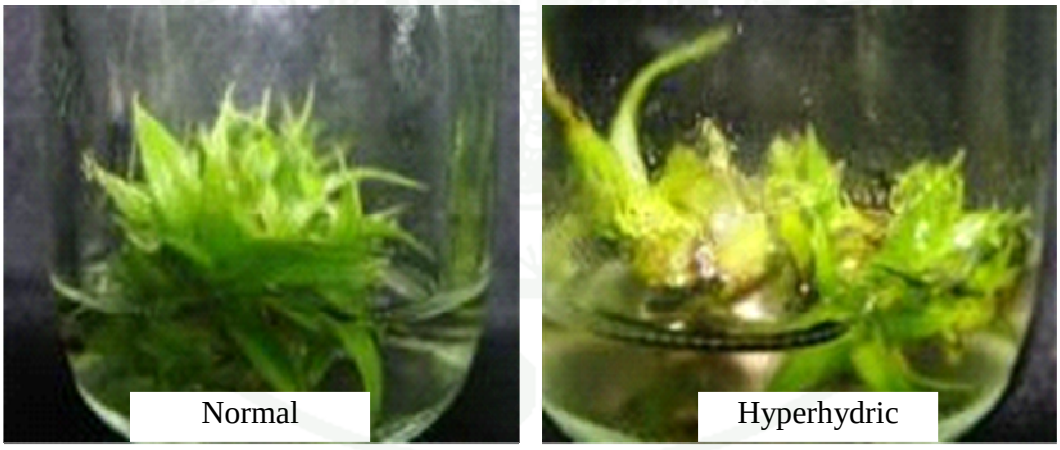

Figure 2 Normal and hyperhydric plantlets of pineapple in the shaking liquid media.

B. Solid media

All media with mT resulted in significantly higher shoot multiplication rate than the control (Table 2). The multiplication rate increased with the mT concentration when mT was individually applied. It is thus worthwhile to conduct experiments using higher concentration of mT in the future. Adding NAA to mT containing media yielded similar effect of the two mT concentrations on multiplication rate. The medium with 5 µM mT alone gave the significantly highest multiplication rate.

Growth and organogenesis *in vitro* are highly dependent on the interaction between naturally occurring endogenous hormone and exogenous plant growth regulators which may be added to the medium (George and Debergh, 2008). The continuous presence of NAA in the multiplication medium was detrimental to growth and multiplication of pineapple shoot-tip culture (Wakasa, 1989). Dolgov *et al*. (1998) reported that the best *in vitro* shoot regeneration of pineapple was obtained when solid media supplemented with (2iP + kinetin) to (NAA + IBA) ratio of 2:1 or 1:5 milligram per liter.

In the solid medium, NAA nllified the multiplication effects of the increase mT concentration. Palni *et al*. (1988) reported that a naturally occurring cytokinin is highly likely liable to degradation by cytokinin oxidases. The enzyme activity could be mediated by NAA. In addition, Nordstrom *et al*. (2004) reported that auxin mediated negative control of the cytokinin pool by mainly suppressing its biosynthesis. In contrast, the effect of cytokinin overproduction on the entire auxin pool in the plant was slower and mediated through altered development.

Table 2 Multiplication rate of 'Patawia' pineapple cultured on solid media with different mT and NAA concentrations for six weeks.

PGR in MS media (µM)		Multiplication (folds)
mT	**NAA**	
0	0	3.25c [1/]
2.5	0	4.00b
5.0	0	6.75a
2.5	2.5	4.25b
5.0	2.5	4.00b
CV (%) 18		

[1/]Means followed by the same letter within a column are not significantly different by Duncan's Multiple Range Test at P=0.05.
PGR= plant growth regulator.

The best mT concentration in the solid media for the shoot multiplication was 5 µM which resulted an average of 6.75 shoots per explant in six weeks (Table 1). This was lower than that of Danso *et al.* (2008) which yield 16.1 fold increase of MD2 pineapple in 6 weeks on a modified MS solid medium with 33.33 µM BA. This dissimilarity might be due to the difference in genotype and/or the type and concentration of cytokinin.

Barboza *et al.* (2004) reported genotypic response to the presence of auxin for shoot proliferation in smooth cayenne pineapple. A medium with 8.88 µM BA and 10.74 µM NAA was best for one genotype while that with 8.88 µM BA was best for another.

C. Temporary immersion system

Meta-topolin containing media resulted in higher multiplication rate than the basal medium. When individually applied, both 2.5 µM and 5 µM mT gave statistically the same result (Table 2). The effect of mT on shoot proliferation was less

pronounced when 2.5 µM NAA was added. More interestingly, the multiplication rate in the media with 5 µM mT and NAA dropped to be the same as that of MS basal medium. In other words, the effect of 5 µM mT on shoot multiplication was nullified by addition of 2.5 µM NAA. The interaction between mT and NAA on pineapple micropropagation, thus, may need an in-depth investigation.

The immersion duration and frequency in temporary immersion system is the most decisive parameter for the efficiency of the system. It provides a highly aerobic system for plant growth (Alvard *et al*., 1993; Etienne and Berthouly, 2002). It also affects nutrient supply and composition of the internal atmosphere in the culture vessel (Jimenez *et al*., 1999). Jackson (2005) stated that an aqueous cover interferes strongly with gas exchange to the outer tissue or cell surface since gas diffusion rates are approximately 10,000 times slower in water than in air. This impact is increased with the depth of the aqueous cover or the inclusion of gel matrices such as agar. Thus, by total submersion, or submersion of the plants too frequently for long periods, gaseous exchange in photosynthesis and respiration was reduced even if there was dissolved oxygen and carbon dioxide in the liquid. Different plants require different flush and rest time for optimal multiplication (Etienne and Berthouly, 2002). Pineapple multiplication and growth was achieved with immersion time of 2 minutes every 3 hours using 200 ml medium in 1 litter twin bottle temporary immersion system (Escalona *et al.*, 1999). On the other hand, pineapple multiplication was attained with immersion time of 6 minutes with every 25 minutes 10 liters vessel by periodical immersion bioreactor (PIB) with few necrotic effects on the shoots (Firoozabady and Gutterson, 2003). The present experiment showed that the single effect of hourly immersion time of 4 and 6 minutes were non-significance on multiplication rate.

Immersion time, however, showed an interaction effect with mT concentration on shoot proliferation. The 4 minute hourly immersion time gave better result in multiplication rate than the 6 minute in both mT concentrations. Thus, future experiment may need to examine the effects of higher concentration of mT with hourly immersion time of 4 minutes.

The hourly immersion time of 4 minutes with the media containing 2.5 or 5 µM mT yielded the highest shoot multiplication rate of 4.25-5 folds in 6 weeks. This was lower than using a modified MS medium with 6.6 µM BA and 2.68 µM NAA in 10 litter periodical immersion bioreactor (PIB) which yielded 5 folds multiplication of a smooth cayenne pineapple in 4 weeks (Firoozabady and Gutterson, 2003). George and Davies (2008) reported that much of the differential behavior of plant cultures on liquid and solid media may be related to differences in oxygen availability. The lowest tolerable level of partial pressures of oxygen may vary according to plant species (Crawford and Braendle, 1996).

Experiment II. **Comparison of Multiplication Rate of Pineapple among the Best Media from the Three *In Vitro* Systems**.

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When the MS medium with 2.5 µM mT for shaking liquid medium and 5 µM mT for both TIS and solid medium were compared for 6 weeks, the shaking liquid medium yielded the highest multiplication rate of 11.96 folds followed by the TIS and the solid media of 10.43 and 8.98 folds, respectively (Table 4). A shaking liquid medium yielded the highest multiplication rate in both the experiment I and the experiment II. However, shoot number per culture from shaking liquid media in the experiment II was lower than that in the experiment I. Whereas solid media and TIS in the experiment II gave higher numbers of shoots than those in the experiment I with the same culture condition. The variation might be due to regeneration capacity of the explants affected by the multiplication cycle (Abdelhamid *et al*., 2008).

The multiplication rate (11.96 folds) obtained from shaking liquid media after 6 weeks of incubation (Table 4) was lower than that in the experiment I, but 6 folds better than the one reported by Firoozabady and Gutterson (2003) and Ika and Ika (2003). It was 2 fold better than the one reported by Be and Debergh (2006) but less than that of Escalona *et al.* (1999). The differences could be attributed to physiological status of the explant, plant growth regulator, subculture frequency and the number of subcultures as well as light intensity (George and Davies, 2008).

Table 3 Multiplication rate of 'Patawia' pineapple using different mT and NAA concentrations and immersion times in temporary immersion system for six weeks.

Immersion time (minute / hr)	PGR (μM)	Multiplication (folds)
4	0 mT + 0 NAA	2.50c
	2.5 mT + 0 NAA	4.25a
	5 mT + 0 NAA	5.00a
	2.5 mT + 2.5 NAA	3.50b
	5 mT + 2.5 NAA	3.25b
6	0 mT + 0 NAA	3.25c
	2.5 mT + 0 NAA	3.50b
	5 mT + 0 NAA	3.75b
	2.5 mT + 2.5 NAA	3.75b
	5 mT + 2.5 NAA	2.50c
Means		
0 mT + 0 NAA		2.87C
2.5 mT + 0 NAA		3.88AB
5 mT + 0 NAA		4.38A
2.5 mT + 2.5 NAA		3.62B
5 mT + 2.5 NAA		2.87C
Means		
4 min / hour		3.70
6 min / hour		3.35
Significance difference		
PGR		* *
Time		ns
Time x PGR		* *
CV (%)		16.7

Means followed by the same letters within a column are not significantly different at P=0.05 level of probability using Duncan's multiple range test.

PGR= plant growth regulator.

ns = Non-significant (P > 0.05); * * = significant (P ≤ 0.05).

The highest shoot production in the liquid media might be favored by a better nutrient uptake than solid medium due to continue contact with culture medium (Almeida *et al.*, 2002, Gupta *et al*., 1981). Escalona *et al.* (1999), however, reported that air-flow twin bottle TIS system with 900 ml vessel yielded the highest pineapple shoot number per explants when compared to liquid and solid media. The availability of CO_2 and O_2 as well as the reduction of C_2H_4 concentration in the system played an important role in shoot multiplication (Escalona *et al.*, 2003). Continuous tissue growth and proliferation was also rapidly limited by the size of the culture container (Debergh *et al*., 1992). In the present experiment, TIS had better multiplication rate than the solid media, but lower than the shaking liquid media. It might be due to shaking liquid media allows parts of the shoots protruded above the surface to expose to the air. Thus, gas exchange for shooting in liquid media might be at about the same level as those in TIS. However, shoots in liquid got longer exposure to the media than TIS.

Rapid rate of plant propagation is depending on the ability to subculture from the proliferated shoots. Be and Debergh (2006) reported that shoot height could vary with the types of the *in vitro* system, species types or other environmental factors. They indicated that the shorter shoot height (less than 2cm) were not suited for subculture or rooting. In this experiment, statistically greater height of shoot was produced in the shaking liquid medium (5 cm) and TIS (4.85 cm) than the solid medium (3.6 cm) (Table 4). However, diameter of shoot was not differently affected by the three *in vitro* systems. This might be because type of culture has varied in their contact areas of the growing shoots, which considerable effect on bud growth.

Fresh weight of shoot was the lowest on the solid media. However, dry weight in the TIS and solid medium was the same. Shoot clusters produced during shoot multiplication in the TIS were nearly spherical. They produced shoots around the central region. This may be due to the reduced development of the leaves in the temporary immersion culture (Escalona *et al.*, 1999).

Table 4 Multiplication and growth of 'Patawia' pineapple shoots under the three *in vitro* systems.

***In vitro* system**	**Shoot multiplication**	**Height (cm)**	**Diameter (cm)**	**Fresh wt (g)**	**Dry wt (g)**
Liquid media	11.96a [1/]	5.00 e [1/]	0.58[ns]	5.77 x [1/]	1.78 s [1/]
Solid media	8.98c	3.60 g	0.58	2.74 y	0.5 t
TIS	9.43b	4.85 f	0.61	5.08 x	0.5 t
CV (%)	14.23	8.58	12.1	11.46	18.04

[1] Means followed by the same letter within a column are not significantly difference by Least Significance Difference test at P=0.05.

TIS= temporary immersion system.

ns = non-significant (P > 0.05).

Experiment III. **Effects of Gamma Irradiation Dose on Pineapple Shoots in the Three *In Vitro* Systems**

After six weeks, all the plantlets under the three culture methods survived from the acute gamma irradiation (Figure 3, 4 and 5). Generally speaking, gamma irradiation had a negative effect on all the growth parameters studied. The growth was reduced with increasing dose of gamma irradiation (Table 5). Statistical analysis revealed that the shoot number from cultures irradiated with 40 and 50 Gy was significantly higher than the shoot numbers obtained from the cultures exposed to 60 and 70 Gy. However, all irradiated cultures had significantly less shoot numbers than the non irradiated cultures (control). Considering shoot height, there was no significant difference between the control and irradiation dose of 40 Gy. However, shoot height was reduced as irradiated dose increased from 50 to 70 Gy. For fresh weight, 40 and 50 Gy significantly reduced fresh weight as compared to the control. The highest gamma irradiation with 70 Gy resulted the lowest fresh weight. Similar to other parameters, increasing irradiation resulted in reducing the dry weight. Irradiated plantlets had significantly lower dry weight than the control. Hence, the shoots treated

with 70 Gy gamma rays were lighter than those treated with 60, 50 or 40 Gy gamma rays with each reduction due to increased irradiation dose being statistically significant. Of all growth parameters, dry weight was the most sensitive parameter to gamma irradiation.

Figure 3 Pineapple plantlets in shaking liquid media, six weeks after acute gamma irradiation.

Figure 4. Pineapple plantlets on solid media, six weeks after acute gamma irradiation.

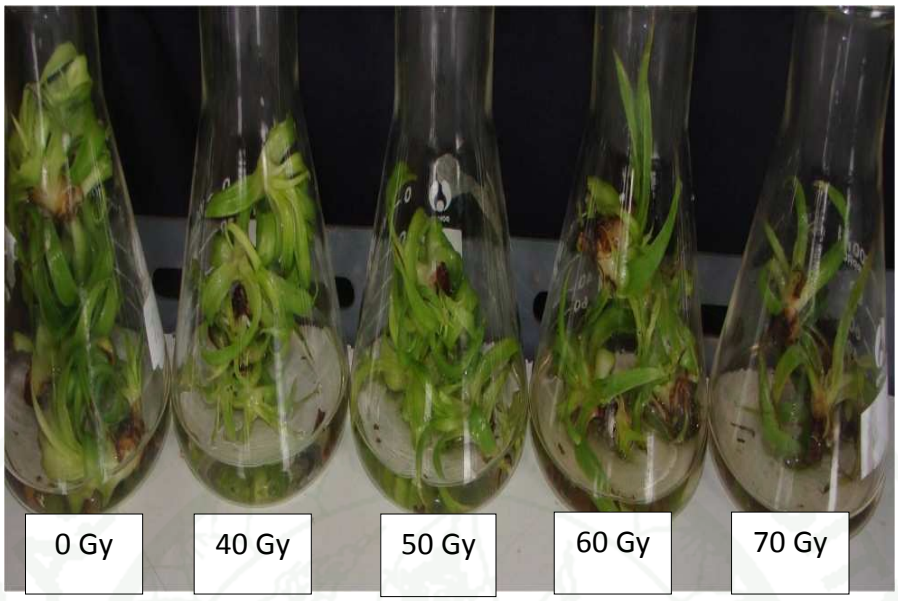

Figure 5 Pineapple plantlets in temporary immersion system, six weeks after acute gamma irradiation.

The negative correlation of gamma irradiation dose and shoot proliferation of *in vitro* pineapple shoot were described by Lapade *et al*. (2002) and Lokko and Amoatey (1998). It was also confirmed here. The highest growth parameters were noted in the non-irradiated cultures while the lowest shoot growth parameters were observed in 70 Gy irradiated. Meyers, (1995) stated that exposure to gamma radiation causes hemolytic fission of hydrogen bond in the water molecules to give H and OH radicals. Hydroxyl radicals react with almost all molecules; e.g. protein, DNA and lipid in living cells and then caused denature, or modify the structure by physiological or chromosomal damage. This could be possibly attributed to less competitiveness of the damaged cells and their progenies (a phenomenon, described as diplontic selection) noted in several *in vitro* mutation induction experiments (Van Harten, 1998).

In vitro systems also varied in their effect on the different growth parameters (Table 5). As compared to other i*n vitro* systems, the shaking liquid media resulted in the highest shoot number (6.2), shoot height (4.48 cm), and fresh and dry weight (4.12 and 1.05 gram, respectively) followed by TIS. The solid media had poor performance for all the growth parameters studied. This might be due to inadequate contact of plant with media in solid culture system (George and Davies, 2008).

Comparing to the experiment I and II, the effects of culture system in the experiment III on non-irradiated treatment differs from the others. The numbers of shoots from both liquid media and TIS in the control gave statistically the same numbers of shoots per explant. The difference was due to the method used to take the shoots out from the TIS culture flask. The folded polycarbonate sheet to hold the explants and narrow mouth of the vessel (Figure 1) made difficulty to take all shoots from the erlenmeyer vessel in the first two experiments. Whereas during the experiment III, the sheet was take out before counting number of shoots. This made higher counted numbers of shoots for TIS in the experiment III than the first two experiments.

The regression equation showed that the GRD_{50} were at 51.52 and 63.8 Gy for TIS and shaking liquid media respectively (Figure 8 and 6). However, gamma irradiation of the highest dose in this experiment (70 Gy) only reduced shoot multiplication in the solid media by 41% (Figure 7). A variation in radiosensitivity was observed after irradiation of shoots from the three *in vitro* systems as measured by the slope of the linear equation of the growth reduction dose of each. The coefficient (the slope) of regression for shoots number in TIS, shaking liquid media and solid media were about 0.0923, 0.0712 and 0.0397, respectively (Figure 6, 7 and 8). Thus, the shoots in TIS and liquid media are more radiosensitive than those on solid media (2.3 and 1.7 times, respectively).

From the slope of the regression equation, the three *in vitro* systems showed variation with respect to the gamma irradiation dosage. The shaking liquid media seems favorable for the growth of the pineapple plantlets as compared with the other culture system. This might give the mutated cell to be plants. Hence, the results from the present experiment suggest that 'Pattawia' pineapple shoots in shaking liquid media is thus recommended for mutation induction with 63.8 Gy of gamma rays. However, this should be done preferably under the same condition to ensure reproducibility of the treatment.

The effects of the gamma rays depends on the culture systems. The shoot numbers on the solid medium was the lowest when compared to the liquid medium and TIS at gamma irradiation of 40 Gy. However, the shoot number in TIS was the lowest when exposed to 70 Gy. The higher number of shoot was yielded from untreated shoots in shaking liquid media and TIS than all the other interaction (Table 5).

A report indicated that LD_{50} for pineapple proliferation was at 45 Gy in liquid media with 20 µM BA (Lokko and Amoatey, 1998). However, LD_{50} for pineapple axillary bud on MS medium supplemented with 8.8 µM BA and 10.74 µM NAA was 40 Gy (Lapade *et al.*, 2002). However, the present study did not get LD_{50} even 70 Gy gamma irradiation was used. The difference among experiments might be

influenced by the physiological condition of the experimental units and the manipulation of the irradiated material before and after treatment as well as the genotype of plant materials (Van Harten, 1998).

Anonymous (1962) reported that there were chemical which can reduce irradiation effects. Some may reduce oxygen tension, while others are attached themselves primarily to the cell structures which make the cell less sensitive. These complex compound formed would guard these sites from the attack of free radicals. Some may also have metabolites which can enhance the repairing effects after irradiation. Since mT has 200 % higher antisenescence activity than BA and zeatin (Strnad *et al.*, 1997; Tarkowska *et al.*, 2003). It might be able reduce radiosensitivity of plantlets. However, it need more research in relation to other internal and external modifying factor.

The effect of the gamma rays depends on the culture systems were also showed on shoot height. The height was generally lowest on solid media. At 40 Gy, the height of those in liquid media and TIS were statistically the same. From 50 Gy and over, the height of those in all the *in vitro* systems were different from each other. The tallest shoot was obtained from the control of shaking liquid media and TIS. Regression analysis revealed the degree of dependence of shoot height (y) of gamma rays (x) in each *in vitro* system. The regression equations were $y= 4.32 – 0.026x$ with ($r^2= 0.53$) for the TIS, $y= 5.08 – 0.012x$ with ($r^2 = 0.64$) for shaking liquid media and $y= 3.53- 0.014x$ with ($r^2= 0.27$) for solid media (Figure 9). When compared among the slope of the three *in vitro* systems, shoots in TIS had the highest radiosensitivity. The solid media had the lowest value of R-square ($r^2=0.27$). It indicated that there may be either other than gamma rays which affected the shoot height of the solid system.

Increasing irradiation dose generally reduced the fresh weight in each *in vitro* system. At all the irradiated doses, fresh weight of the solid media was the lowest. At 0 and 60 Gy, fresh weight of the shoots in the TIS was lower than those in liquid medium. Whereas at the other dose, there was no significant difference between liquid

media and TIS. Regression analysis revealed that the degree of dependence shoot fresh weight (y) with gamma rays dose (x) in each *in vitro* system. Y= 5.20 – 0.032x with (r^2= 0.87) for the TIS, Y= 5.68 – 0.35x with r^2 = 0.72) for shaking liquid media (Figure 10). Thus, shoot in TIS had higher radiosensitivity than shaking liquid media.

Considering dry weight, the effects of gamma rays was also depend the types of the *in vitro* systems. At 40 Gy, dry weight of shoots in the liquid medium were significantly higher than in TIS and solid media. The highest dry weight was from the non-irradiated shoots of liquid media while the lowest was recorded from TIS and solid media irradiated with 70 Gy. At 60 or 70 Gy, dry weight of shoot in the solid media and TIS were no significance difference. Regression analysis revealed the degree of dependence shoot dry weight (y) with gamma rays dose (x) in each *in vitro* system. Y= 0.79 – 0.007x with (r^2= 0.87) for the TIS, Y= 0.43 – 0.004x with (r^2 = 0.84) for shaking liquid media (Figure 11). When compared between slopes of the two *in vitro* systems, shoots in TIS had the higher radiosensitivity than liquid media.

As R-square was very low (< 0.25), for the regression equation of fresh and dry weight in solid media, it is not worth to compare with other culture systems. The low R-square indicated that weak prediction of the dependent variable (fresh or dry weight) from the regression model. However, there is no clear explanation why the R-square was very low. It might be important to study further with more numbers of samples in the future.

Oxygen acts as a modifying factor for gamma rays by reacting with all chemical compounds and affects the reaction mechanisms present in the system (Van Harten 1998). Nilan *et al*. (1965) reported that the presence of oxygen during irradiation appears to be increased the response of biological systems to radiation whereas in the absence of oxygen the radiation damage reduced. The magnitude of radiation lesions can be altered by changes in oxygen tension after irradiation. The *in vitro* system are reported to affect the dissolved oxygen. The oxygen concentration in plantain culture was 15.1% for solid medium while temporary immersion system had

19.3% oxygen (Roels *et al.*, 2005). The oxygen variation in the *in vitro* systems might thus be responsible to the radiosensitivity difference in the present experiments.

Correlations between the physiological status of plants and their radiosensitivity are often determined by the water content of the tissue (Meyers, 1995). Furthermore, treatment of the irradiated materials in vessels containing some water prior of gamma rays treatment enhance irradiation efficiency (Predieri and Gatti, 2000). In the present experiment, the higher radiosensitive culture system, TIS, had more moisture content (85%) than shaking liquid media and solid media (about 70% each).

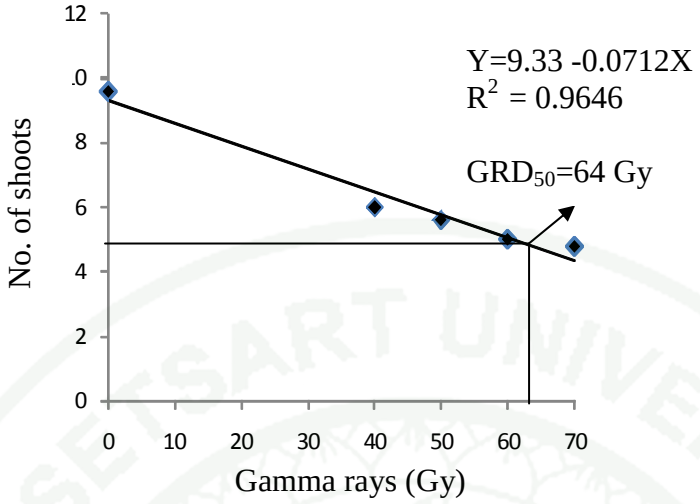

Figure 6 Response curve of *in vitro* pineapple shoots in shaking liquid medium to gamma rays, six weeks after acute gamma irradiation.

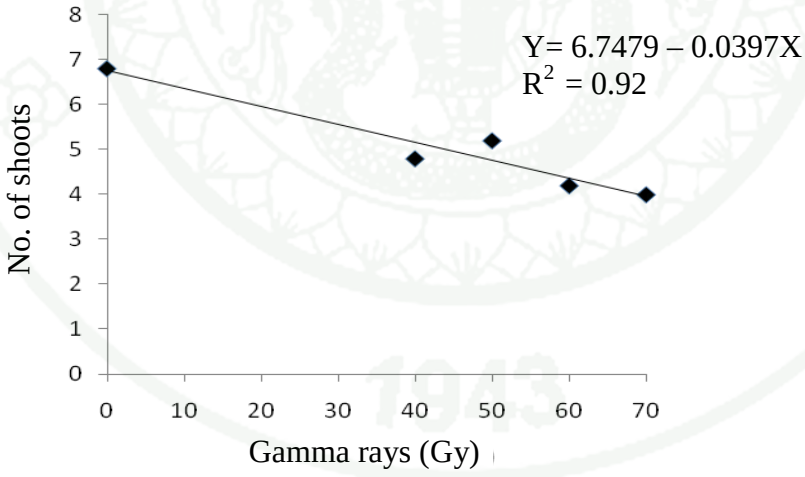

Figure 7 Response curve of *in vitro* pineapple shoots on solid medium to gamma rays, six weeks after acute gamma irradiation.

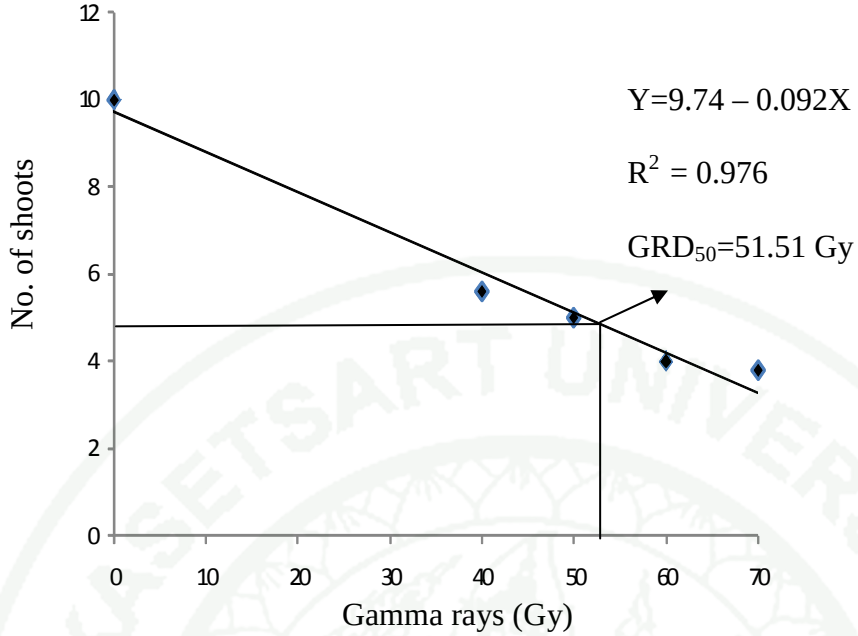

Figure 8 Response curve of *in vitro* pineapple shoots in temporary immersion system to gamma rays, six weeks after acute gamma irradiation.

Table 5 Growth of 'Patawia' pineapple shoots in the liquid media, solid media and TIS, six weeks after acute gamma irradiation.

In vitro systems	Gamma ray (Gy)	Shoot number	Height (cm)	Fresh Wt. (g)	Dry Wt. (g)
Liquid	0	9.60a [1/]	5.00a [1/]	5.77a [1/]	1.76 a [1/]
	40	6.00c	4.60ab	4.04cd	1.21 b
	50	5.60cd	4.29b	3.73cde	0.65 def
	60	5.00def	4.32b	3.84dc	0.89 cd
	70	4.80ef	4.17b	3.22efg	0.76 de
Solid	0	6.80b	3.10cd	1.55ij	0.46 fghi
	40	4.80ef	3.50c	1.85hi	0.56efgh
	50	5.20cde	3.60c	2.77gh	0.82de
	60	4.42ef	2.45e	1.23j	0.12kj
	70	4.00ef	1.85f	0.84k	0.11 k
TIS	0	10.0a	4.85a	5.08b	0.76de
	40	5.60cd	4.30b	4.34c	0.61defg
	50	5.00def	3.35c	3.40de	0.36ghij
	60	4.00fg	3.30c	3.26ef	0.31hijk
	70	3.80g	3.15c	2.89efg	0.27ijk
LSD		0.74	0.48	0.438	0.28
Gamma rays (Gy)					
0		8.8 A	4.32A	4.14A	0.99A
40		5.47B	4.13A	3.41B	0.79B
50		5.27B	3.75B	3.30B	0.61C
60		4.4C	3.56C	2.78C	0.44D
70		4.2C	3.06D	2.32D	0.38E
In vitro systems					
Liquid		6.2A	4.48A	4.12A	1.05A
Solid		5.00C	2.90C	1.65C	0.42C
TIS		5.68B	3.79B	3.80B	0.46B
Significance difference					
In vitro system x Rays		**	**	**	**
In vitro systems		**	**	**	**
Gamma rays		**	**	**	**
CV (%)		10.36%	10.26%	10.87%	3.47%

[1/]Means followed by the same letter within a column (small letter for mean of interaction and capital letter for single mean effects) are not significantly different using Least Significance Difference test (at P=0.05). * * = significant (P≤ 0.05).

Stimulation of tissue culture response at low irradiation doses has been demonstrated in banana and sugarcane and other crops (Kulkarni *et al.*, 1997; Charbaji and Nabulsi, 1999). In the present experiment, solid media with the irradiation dose of 50 Gy was also show increment in their fresh and dry weight of pineapple shoots (Table 5).

In general, the negative effects on plant morphology observed in this study were due to the irradiation which induced disturbances in the normal physiological functioning. Although it appears that the radiosensitivity is a complex function of several factors such as physiological status of the plant tissue, degree of differentiation of tissue, stage and rate of growth (dividing or non-dividing), the irradiation dose and rate, and also, the genetic architecture of the species (Karmarkar *et al*., 2005).

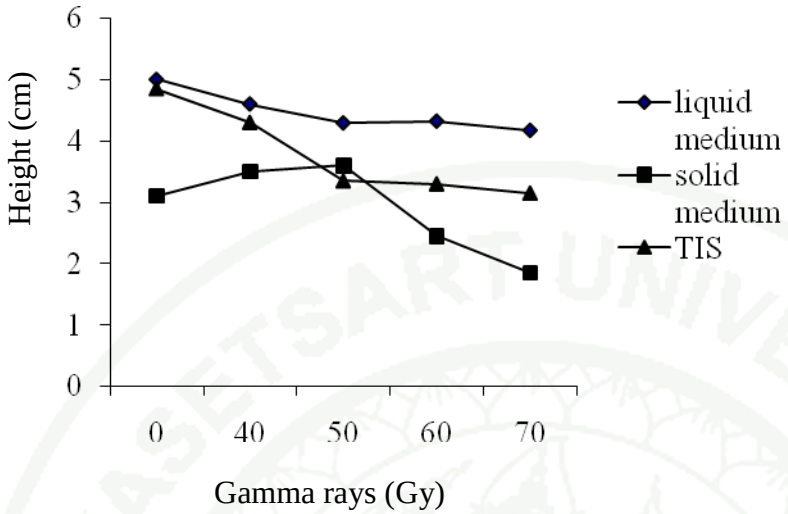

Figure 9 Height of pineapple shoots under three *in vitro* systems, six weeks after acute gamma irradiation

	Shaking liquid media	Solid media	TIS
Regressions	Y= 5.08-0.012x	Y=3.53-0.014x	Y=4.32-0.026x
R-square	0.64	0.27	0.53
Probable	**	**	**
Correlation	-0.88		

* * = significant ($P \leq 0.05$)

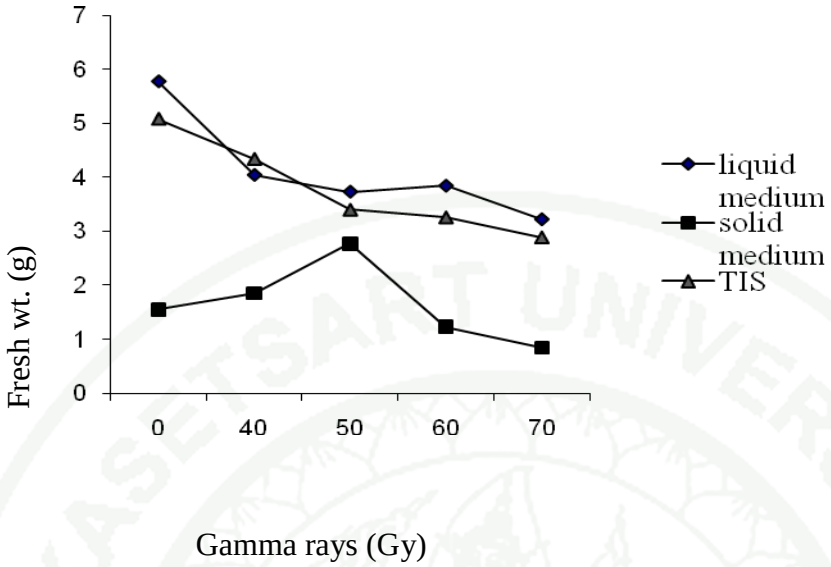

Figure 10 Fresh weight of pineapple shoots under three in vitro systems, six weeks after acute gamma irradiation

	Shaking liquid media	Solid media	TIS
Regressions	Y= 5.68- 0.35x	Y=1.91 - 0.006x	Y=5.20 -0.032x
R-square	0.72	0.05	0.87
Probable	**	**	**
Correlation	-0.88		

* * = significant ($P \leq 0.05$)

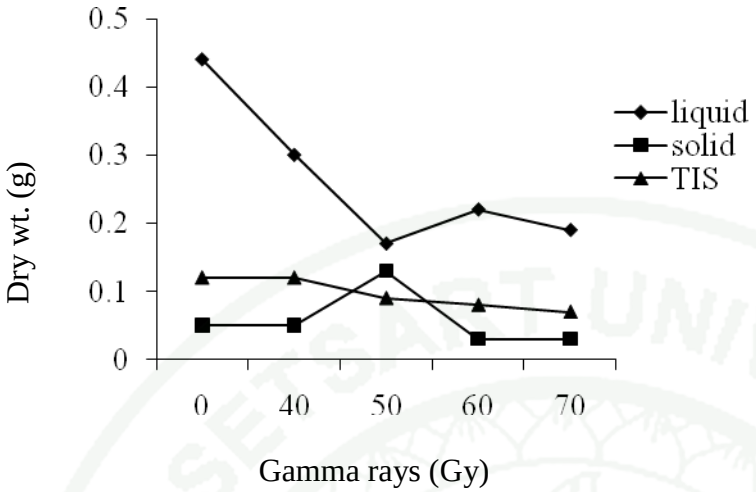

Figure 11 Dry weight of pineapple shoots under three *in vitro*, system, six weeks after acute gamma irradiation.

	Shaking liquid media	Solid media	TIS
Regressions	Y=0.43 -0.004x	Y=0.61-0.004x	Y=0.79-0.007x
R-square	0.87	0.15	0.87
Probable	**	**	**
Correlation	-0.88		

* * = significant ($P \leq 0.05$)

SUMMARY AND CONCLUSIONS

Multiplication rate of pineapple after 6 weeks in three *in vitro* systems with different mT and NAA concentration were studied. The optimal mT concentration in the shaking liquid media was at 2.5 µM. It resulted in higher number of shoots per explant (15.75) with relatively healthy plantlets than the basal media. The mT concentration for the solid media was 5 µM, with an average of 6.75 shoots per explant. In TIS, both, 2.5 µM and 5 µM mT gave statistically the same result. However, 4 minute hourly immersion time with the medium 2.5 or 5 µM mT in medium gave the best shoot multiplication of 4.25- 5 folds. For the hourly immersion time, 4 and 6 minutes gave statistically the same effect on *in vitro* shoot multiplication. NAA showed some antagonistic effects to mT on multiplication rate as well as the hyperhydricity control.

When the best media from each culture systems were compared for 6 weeks, the shaking liquid media with 2.5 µM mT had the highest multiplication rate of 11.96 folds followed by the TIS and the solid media with 5 µM mT of 10.43 and 8.98 folds, respectively. This might be due to a better contact with the liquid media than with the solid media. Moreover, most shoot in the shaking liquid media exposed to the air above the media. The gaseous available in TIS and the shaking liquid media thus virtually the same.

Pineapple shoots from the three *in vitro* systems, i.e. solid, shaking liquid media and TIS, were subjected to different gamma rays dose. The GRD_{50} from multiplication were at 51.52 and 63.8 Gy under TIS and shaking liquid media, respectively. The tested dose range was unable to reach GRD_{50} for the solid medium.

This investigation suggested that *in vitro* mutagenesis of ‘Patawia’ pineapple should be executed using in shaking liquid MS media with 2.5 mT and 63.8 Gy acute gamma rays irradiation.

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APPENDIX

Appendix Tables 1 ANOVA for pineapple shoot multiplication in shaking liquid media with different mT and NAA concentrations.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Treatment	4	187.7	46.925	11.45	0.0002	11.2	0.75
Error	15	61.50	4.10				
Corrected Total	**19**	**249.20**					

Treatment = mT and NAA concentrations.

Appendix Tables 2 ANOVA for pineapple shoot multiplication on solid media with different mT and NAA concentrations.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Treatment	4	28.70	7.175	10.50	0.0003	4.45	0.74
Error	15	10.25	0.683				
Corrected Total	**19**	**38.95**					

Treatment = mT and NAA concentrations.

Appendix Table 3 ANOVA for pineapple shoot multiplication in TIS with different immersion time, mT and NAA concentrations.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Time	1	1.225	1.225	1.86	0.1827	3.53	0.51
Treatment	4	13.600	3.400	5.16	0.0028		
Time x Treatment	4	5.400	1.350	2.05	0.1124		
Error	30	19.750	0.6583				
Corrected Total	**39**	**39.975**					

Treatment = Types of culture system.

Appendix Table 4 ANOVA for pineapple shoot multiplication in shaking liquid media, solid media and TIS, six weeks after acute gamma irradiation.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Dose	4	206.480	51.620	151.82	<.0001	5.63	0.93
Treatment	2	18.1067	9.053	26.63	<.0001		
Treatment x Dose	8	22.560	2.820	8.29	<.0001		
Error	60	20.400	0.340				
Corrected Total	**74**	**267.547**					

Treatment = Types of culture system.

Appendix Tables 5 ANOVA for pineapple shoot height in shaking liquid media, solid media and TIS, six weeks after acute gamma irradiation.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Dose	4	16.505	4.126	28.31	<.0001	3.72	0.864
Treatment	2	31.204	15.60208	107.04	<.0001		
Treatment x Dose	8	7.821	0.97765	6.71	<.0001		
Error	60	8.746	0.14576				
Corrected Total	**74**	**64.268**					

Treatment = Types of culture system.

Appendix Table 6 ANOVA for fresh weight of pineapple shoot in shaking liquid media, solid media and TIS, six weeks after acute gamma irradiation.

Source	Df	Sum square	Mean Square	F Value	P > F	Mean	R-square
Dose	4	28.3140	7.078505	58.86	<.0001	3.19	0.95
Treatment	2	90.1240	45.062021	374.72	<.0001		
Treatment x Dose	8	17.2410	2.155126	17.92	<.0001		
Error	60	7.2152	0.120254				
Corrected Total	**74**	**142.8942**					

Treatment = Types of culture system.

Appendix Table 7 ANOVA for dry weight of pineapple shoots in shaking liquid media, solid media and TIS, six weeks after acute gamma irradiation.

Source	**Df**	**Sum square**	**Mean Square**	**F Value**	**P > F**	**Mean**	**R-square**
Dose	4	3.865272	0.966318	1932.64	<.0001	0.64347	
Treatment	2	6.334763	3.167381	6334.76	<.0001		
Treatment x Dose	8	2.884064	0.360508	721.02	<.0001		
Error	60	0.03000	0.0005				
Corrected Total	**74**	**13.114099**					

Treatment = Types of culture system.

Appendix Table 8 Stock solution for MS media preparation.

Code	Chemicals	Stock solution (g/L)	Stock solution to prepare 1 liter of media (ml)
MS-1 20x	Ammonium nitrate: NH_4NO_3	33.0	50
	Potassium nitrate: KNO_3	38.0	
	Magnesium sulfate: $MgSO_4.7H_2O$	7.4	
	Calcium Chloride: $CaCl_2.2H_2O$	8.8	
	Potassium dibasic phosphate: K_2HPO_4	3.4	
MS-2 100x	Manganese sulfate: $MnSO_4.4H_2O$	1.69	10
	Cobalt chloride: $CoCl_2.6H_2O$	0.0025	
	Zinc sulfate: $ZnSO_4.7H_2O$	0.86	
	Copper sulfate: $CuSO_4.5H_2O$	0.0025	
	Boric acid: H_3BO_3	0.62	
	Sodium molybdate: $Na_2MoO_4.2H_2O$	0.25	
	Potassium Iodide : KI	0.083	
MS-3 100x	Na_2EDTA	2.78	10
	$FeSO_4.7H_2O$	3.726	
MS-4 100x	Glycine	0.2	10
	Thiamine HCl	0.01	
	Pyridoxine HCl	0.05	
	Nicotinic acid	0.05	

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