International Journal of Agricultural Technology 2019 Vol. 15(1): 63-74
Available online http://www.ijat-aatsea.com
ISSN: 2630-0613 (Print) 2630-0192 (Online)

In vitro effect of gamma irradiation and plant growth regulators (PGRs) for induction and development of *Stylosanthes hamata* cv. Verano

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Ngoenngam, L., Pongtongkam, P., Arananant, J., Poeaim, S. and Poeaim, A. (2019). *In vitro* effect of gamma irradiation and plant growth regulators (PGRs) for induction and development of *Stylosanthes hamata* cv. Verano. International Journal of Agricultural Technology 15(1): 63-74.

Abstract Pasture legumes was developed by tissue culture technique using gamma irradiation. Seeds of *Stylosanthes hamata cv*. Verano were received 7 doses of radiation (5, 10, 15, 20, 25, 30 and 45 Krad) and control (without irradiation) cultured on MS medium. Free Plant Growth Regulators (PGRs) and 30 g/L sucrose treatment found that 30 Krad decreased the percentage of shoot and root length of 31.7% and 20.51% respectively in 30 days. While seeds without irradiation were 100% germination. The radiation doses was reduced the growth rate by half in 7 days $GR_{50(30)}$ at 39.82 Krad. The lethal dose (LD_{50}) of *Stylosanthes hamata* cv. Verano was 26.14 Krad and the higher irradiation level increased death rate. Seeds on MS medium with 3 mg/L of *meta*-Topolin (*m*T) resulted at 60 days after irradiation which higher than 50 % in all treatments. Moreover, every dose of gamma rays stimulated shoots and roots after 60 days.

Keywords: gamma irradiation, lethal dose (LD_{50}), *Stylosanthes hamata* cv. Verano

Introduction

The most of livestock animals in Thailand are beef cattle and dairy cattle, fed by concentrate and rough food. Rough foods are grasses and beans. Dairy are fed cows by rough food up to 15% of their total daily intake. When the dairy cow receives lower coarse feed than 15%, the milk fat content will decrease, and it effects on digestion system in the rumen and animal health (Thadsri, 1997). Nowadays, Department of Livestock, Thailand has introduced 285 species of beans for study. However, there are only 4 species that produce seed as well are *Centrosema pascuorum* cv. *Cavalcade*, *Desmanthus virgatus*, *Stylosanthes hamata* cv. Verano and *Stylosanthes guianensis*, CIAT 184, which has a seed yield are 67.2, 39.0, 39.7 and 33.0 tons, respectively.

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Hamata beans (*Stylosanthes hamata* cv. Verano), a native bean from Venezuela in South America, was been stuide by Khonkhean University since 1970 by Dr. H.M. Shelton and Dr. L.R. Humphreys, Queenlands University, Australia. (Boonruang *et al*., 2012). Hamata beans can be grown in a generally general, most cultivated in the Northeast, Thailand, protein content and seed yield are quite high, resistant to animals grazing and it is no report of cattle bloating (Bureau of Animal Nutrition Development, 1982). Khon Kaen university reported the comparative test found that Hamata beans produced higher productivity more than. Another bean in *Stylosanthes* type. This report is similar to (Topark-Ngarm, 1979) reported the various methods to develop the maximum product of seed. For example, the study of appropriate time to release sheep or goat for grazing before seeds harvesting (Wilaipon and Humphreys 1976; Wilaipon and Humphreys, 1981) or period to truncate the bean before seeds harvesting (Wilaipon and Humphreys, 1979).

The feeding beans was done by tissue culture technique with gamma irradiation. Makthong (2013) reported that induction to mutate in (*Medicago sativa* L.) beans by gamma ray. Seed were received doses of radiation of 100, 200, 300, 400, 500, 600, 700, 800, and 900 Gy, the result showed that seeds with 100 Gy of gamma rays decreased the percentage of shoot at 39.2% while seed without gamma irradiation was percentage of shoot at 94.4%, and LD_{50} was 100 Gy of gamma rays which suitable for mutation. Benslimani and Khelif (2009) reported that induction of mutation in *Arachis hypogaea* L. by ^{60}Coirradiation with doses were 50, 100, 150, 200, 300 and 500 Gay. Seeds with 450 Gay of cobalt rays decreased percentage of shoot and root length about 46.5% and 59.0% respectively. Mba *et al*. (2010) suggested doses of gamma irradiation from 0 to 600 Gy should be using for genotype test to choose the optimal treatment condition. Horn and Shimelis. (2013) reported on the various doses of radiation at 0, 100, 200, 300, 400, 500 and 600 Gy in Cowpea (*Vigna unguiculata*) by ^{60}Co irradiation. The result showed that genotype of Nakard tolerated at 200 Gy, and provided percentage of germination at 43.33%. The optimum dose of 150 Gy made LD_{50} of Nakard. Tissue culture technology play a huge role to improve plant variety and combination with gamma irradiation to induce mutation. The aim of experiment was to study the effect of gamma irradiation and plant growth regulators of Hamata beans to induce drought tolerant and salt tolerant.

Materials and methods

Plant material

Seeds of Hamata beans (*Stylosanthes hamata* cv.Verano) were supported by Feed and Forage Analysis Section, Animal Nautrition

Division, Department of Livestock Development, Pathumthani Province, Thailand.

Gamma Radiation

The gamma radiation was carried out at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University, Bangkok, Thailand. In acute irradiation, seeds were irradiated with gamma radiation from Cs-137 using a research irradiator (Mark I) at doses of 0 (control) 5, 10, 15, 20, 25, 30, and 45 Krad. The 0 Gy using as a comparative control. The explants were surface sterilized by using 70% (v/v) ethanol for 2 min, 15%(w/v) sodium hypochlorite solution with 2 to 3 drops of Tween 20 for 15 min, three times rinse in distilled water for 5 min per times. MS medium (Murashike and Skoog, 1962) is used as basal medium with salts and vitamins, added 30 g/L sucrose, 2.6 g/L phytagel, adjusted pH of the medium to 5.8 using 0.5 N NaCl, autoclaved at 121 ℃ for 15 min. The cultures were incubated at 25 ± 2 °C under cool white fluorescent light (27 µmol $m^{-2}s^{-1}$) with 16 h light/8 h dark photoperiod. GR_{50} (50% growth reduction), a dose can decrease 50% of seeding height. It was determined for all treatments by measuring the seedling height, shoots and root length after 30 days $GR_{50(30)}$. After 30 days, data were calculated the germination percentage (GP) using formula 1 (Dezfuli *et al*., 2008), the lethal dose 50 percentage (LD_{50}) was used by the following formula 2 (Bashir, 2012).

$$GP = \frac{\text{No. of seeds germinated at 30 days after germination}}{\text{No. of seed control}} \; x\ 100$$

$$\text{Survival \%} = \frac{\text{N0. of seedlings survived}}{\text{N0. of seeds germinated}} \; x\ 100$$

Effect of growth regulators on the growth of bean seeds after irradiation

Seeds with eight doses of radiation, 0 (control) 5, 10, 15, 20, 25, 30, and 45 Krad, were cultured on MS medium added 30 g/L sucrose, 2.6 g/L phytagel and 3 mg/L meta-Topolin (*m*T). The pH of the medium was adjusted to 5.8 using 0.5 N NaCl, autoclaved at 121 ℃ for 15 min. The cultures were incubated at 25 ± 1 °C under cool white fluorescent light (27 µmol $m^{-2}s^{-1}$) with a 16 h light, 8 h dark photoperiod, and measured LD_{50} and GR_{50} after 30 days.

Data analyses

All treatments were repeated three times. ANOVA was computed using SPSS v17. The data for each stage of the experiment was analyzed using one-way ANOVA and followed by Duncan's multiple range test (DMRT) for mean comparison at p-values 0.05.

Results

Effects of gamma irradiation on germination

The effect of gamma irradiation on Hamata seeds were irradiated with 0, 5, 10, 15, 20, 25, 30 and 40 Krad, surface sterilized seeds were cultured on MS agar without plant growth regulators. In general, increasing the gamma dose caused negative effect on plant development as it was indicated by the reduction height, shoot and root lengths at 30 days. The germination response of Hamata beans against irradiation doses were given the linear equations: y = -1.5723x + 112.61 (Fig 1). The highest of $GR_{50(30)}$ values based on the height of Hamata was 39.82 Krad.

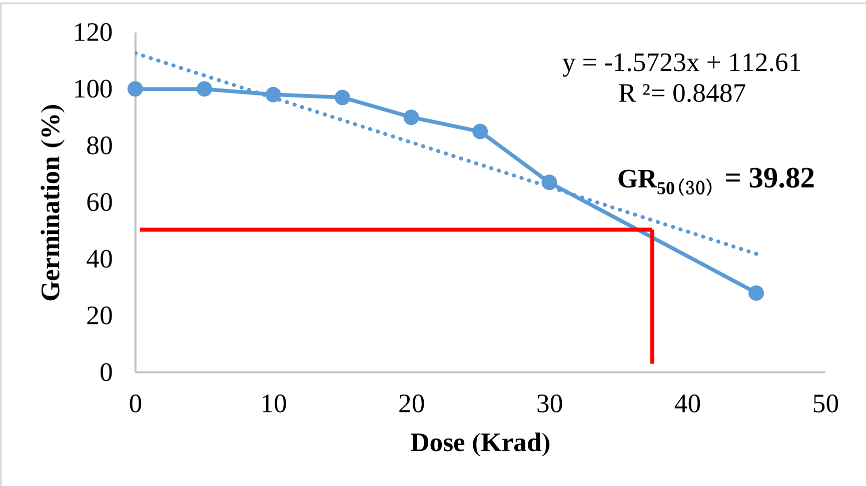

Figure 1. GR_{50} at 30 days of *Stylosanthes hamata* cv. Verano plants treated with different dose of gamma rays

Seed germination, percentage of shoot initiation, percentage of survival, shoot and root lengths of 30 days seedings was shown in Table 1. The percentage of seed germination gradually decreased as gamma doses increased to 45 Krad in 30 days. The highest percentage of seed germination was 60% at 5 Krad. The highest percentage of shoot initiation shoot and root length at 5 Krad gamma dose of 100%, which were 0.625 cm and 0.344 cm, respectively. The 45 Krad was lowest of 28 %, which shoot and root lengths were 0.114 cm and 0.050 cm, respectively. Roots were stopped growing and cotyledon wilted (Table 1, Fig 2).

Table 1. Total number of initial seeds, germination rate, survival rate, shoot and root lengths acutely exposed to different doses of gamma radiation on MS medium at 30 days

Gamma Dose (Krad)	The number of initial seeds	Germination (%)[1/]	Survival (%)[2/]	Shoot length (cm)	shoot response %	Root length (cm)	Root response %
Control	60	60(100)	60(100)	0.680±0.70 [a]	100	0.546±0.10[a]	100
5	60	60(100)	60(100)	0.625±0.66[b]	91.91	0.344±0.35[b]	63.00
10	60	59(98)	56(93)	0.423±0.40[c]	62.21	0.348±0.15[b]	63.73
15	60	58(97)	48(80)	0.367±0.52[d]	53.97	0.311±0.75[b]	56.95
20	60	54(90)	44(73)	0.353±0.50[de]	51.91	0.257±1.27[c]	47.07
25	60	51(85)	34(57)	0.310±1.10[e]	45.58	0.114±0.65[d]	20.88
30	60	40(67)	17(28)	0.214±1.45[f]	31.47	0.112±0.64[d]	20.51
45	60	17(28)	4(6)	0.114±0.57[g]	16.76	0.050±0.56[e]	9.15

Table 2. Total number of initial seeds, germination rate, survival rate, shoot and root lengths acutely exposed to different doses of gamma radiation on MS medium with 3 mg/L of *m*T. The effects were investigated for 60 days

Dose (Krad)	The number of initial seeds	Germination (%)[1/]	Survival (%)[2/]	Shoot length (cm)	shoot response (%)	Root length (cm)	Root response (%)
***m*T3(control)**	60	60(100)	60 (100)	0.880±1.44[a]	100	0.351±1.15[a]	100
5	60	60(100)	60(100)	0.829±0.05[a]	94.20	0.206±0.35[b]	58.68
10	60	59(98)	59(98)	0.695±0.60[b]	78.97	0.225±1.12[b]	64.10
15	60	58(97)	51(85)	0.555±1.27[c]	63.06	0.243±0.75[b]	69.23
20	60	59(98)	48(80)	0.414±1.25[d]	47.04	0.236±1.52[b]	67.23
25	60	55(92)	47(78)	0.464±0.70[d]	52.72	0.232±0.11[b]	66.09
30	60	49(81)	31(52)	0.595 ±0.50[c]	57.38	0.326±0.30[a]	92.87
45	60	34(57)	30(50)	0.552±0.26[c]	62.72	0.321±0.35[b]	65.81

[a-d] Means in same column with different letters are significantly different at P < 0.5 according to DMRT using one-way ANOVA
[1/] Seed germination means germinated seeds out of total seed number.
[2/] Shoot growth initiation means developed shoots out of total seed number

Figure 2. *In vitro* seed germination, shoot and root length of *Stylosanthes hamata* cv. Verano seeds irradiated with 0, 5, 10, 15, 20, 25, 30 and 40 Krad on MS medium at 30 days

The apical meristems were yellow, observed at 26 days death. The leaves cannot grow due to severe physical damage. The survival response of Hamata against irradiation doses was given the linear equations:

$$y = -2.3151x + 110.53 \text{ (Fig 3)},$$

while $LD_{50(30)}$ was obtained at 26.14 Krad.

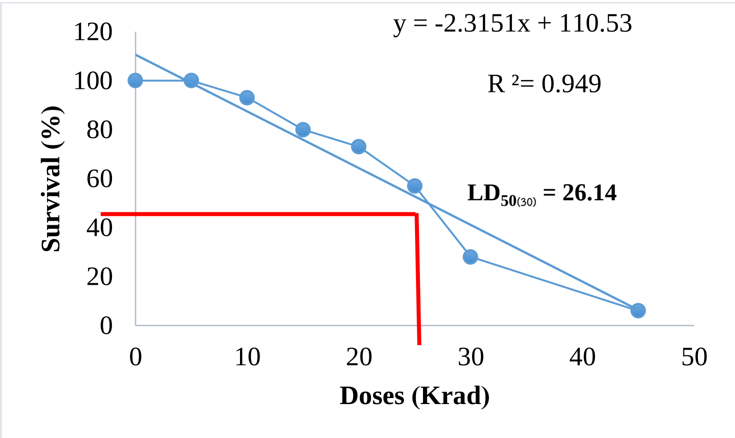

Figure 3. LD_{50} at 30 days of *Stylosanthes hamata* cv. Verano plants treated with different dose of gamma rays

Effect of gamma irradiation on meta-Topolin (mT)

The irradiation activated in the MS medium added 3 mg/L meta-Topolin (*m*T) for 60 days. Hamata showed a growth rate over 50 percent in all treatments (Table 2). The treatments of 0, 5, 10, 15, 20, 25, 30 and 45 Krad were germinated at 100, 100, 98, 85, 80, 78, 52 and 50 percentage, respectively. Seeds obtained gamma rays at 30 and 40 Krad with an average shoot length were 0.595 and 0.552 cm, respectively. The mean length was higher than seeds without hormone which cultured on same medium. Average root length of the control plants and the 30 Krad were 0.351 and 0.326 cm, respectively. All treatments caused calli around the roots (Fig 4). The results showed the survival percentages at 60 days after irradiation were higher than 50 % in all treatments.

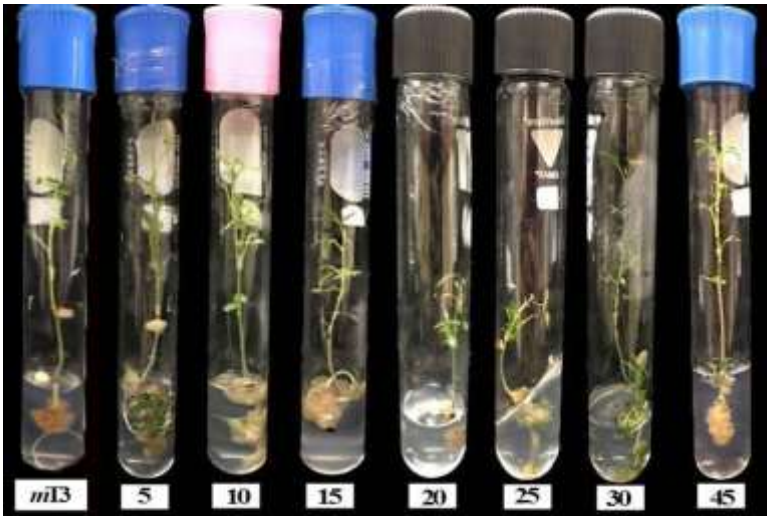

Figure 4. In *vitro* of seed germination shoot and root length of *Stylosanthes hamata* cv. Verano seeds irradiated with 0, 5, 10, 15, 20, 25, 30 and 40 Krad on MS medium added 3 mg/l of *m*T at 30 days

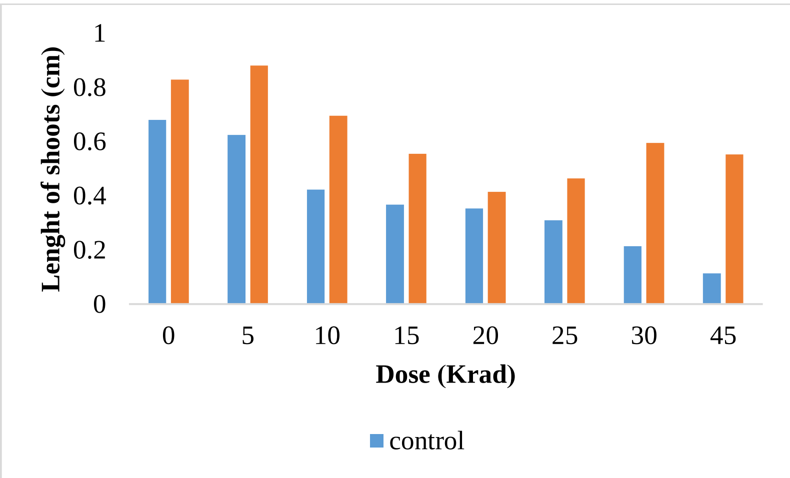

Figure 5. Effects on the length of shoots of *Stylosanthes hamata* cv. Verano by gamma irradiation

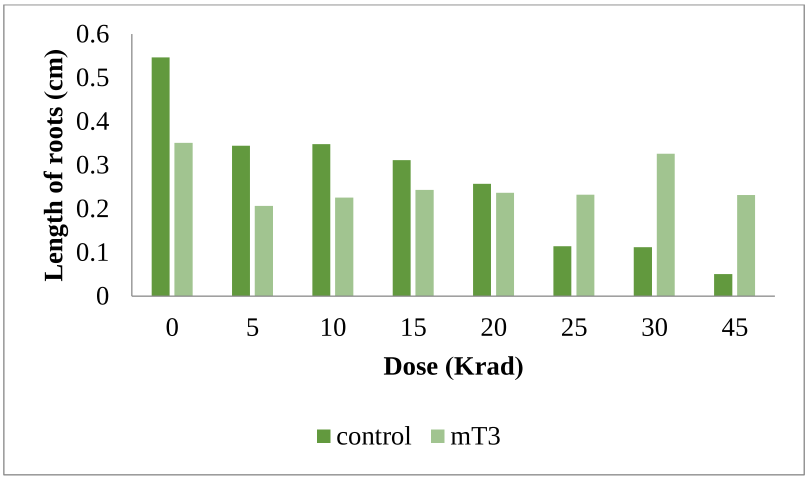

Figure 6. Effects on the length of roots of *Stylosanthes hamata* cv. Verano by gamma irradiation

A morphological investigation was conducted for 30 days to compare the heights of seeds on MS medium with 3 mg/L of *m*T and free PGRs. In general, the results enhanced the degree of seeds on MS medium with 3 mg/L of *m*T from 0 to 45 Krad with increased in seedling height when compared with those culture on MS medium without hormone (Fig 5). The root length of the plantlets significantly increased in the second week of culture. Plantlets irradiated at 30 and 45 Krad changed in height in all doses when compared with those culture on MS media without hormone (Fig 6).

Discussion

The effect of gamma irradiation on Hamata seeds were irradiated with 0, 5, 10, 15, 20, 25, 30 and 40 Krad .The higher of $GR_{(50)30}$ values based on the plant height of Hamata were 39.82 Krad or around 400 Gy. These results are similar to Kimno and Chepkoech (2014) studied on the induction of mutation using gamma rays on *Sorghum bicolar* L. Seed beans were treated 200, 400 and 600 Gy. Dose of gamma rays at 406 Gy (40 Krad) decreased rate of germination. Ramazan *et al*. (2016) varied doses of ^{60}Co irradiation at 0, 50 100 150 200 and 250 Gy on *Lathyrus chrysanthus* Boiss. After 14 days, 250 Gy (Krad) affected to seed germination of 14.3%, shoot and root length were 0.5 and 1.3 cm, respectively. Seed of Hamata beans irradiated at 45 Krad (450 Gy), seed germination was 28.0%, shoot and root length were 0.114 and 0.05 cm. From the result showed that low dose of irradiation had no effect on germination rate (IAEA, 1979). Survival, sprouting ability, height and the number of plant organs were affected by higher dose of gamma rays (Nwachukwu *et al*., 2009; Devi and Mullainathan, 2012). In high dose of gamma irradiation, the growth of root was stopped, cotyledon was wilted, characteristics of seed changed both in morphology and genetic. Survival plant can repair itself after irradiated with gamma rays and survive depending on dose of irradiation (Ahloowalia, 1998). These changes are the result of genetic was destroyed. It also creates free radicals that can change the structure of the plant cells, physiological and biochemical processes of the plant (Marcu *et al*., 2013). Hamata beans after 26 days cultivated, apical meristems were changed green to yellow and eventually die. The Leaf stop growing caused by physical damage, which usually resulted in halting of apical meristem or root. The changes in external genes of the nucleus, it can not transfer to next generation (Vos *et al*., 1995; Lee *et al*., 2002).

The LD_{50} value of Hamata beans with gamma irradiation on MS basal agar medium without PGRs at 26.14 Krad (260 Gy) was created. Rajarajan *et al*. (2016) reported on varied radiation doses of gamma rays at 150, 200, 250, 300 and 350 Gy in rice cultivar ADT (R) 47. The result showed LD_{50} value after irradiated at 229 Gy. Gang *et al*. (2007) studied on *Narcissus tazetta* received the highest dose of radiation, survival rate of the plants was significantly reduced.

Seeds of *Stylosanthes hamata* cv. Verano were received 8 doses of radiation on MS medium with 3 mg/L, meta-topolin (*m*T). The results showed the percentages of survival after 60 days with radiation higher than 50 % in all treatments and $GR_{50(60)}$ higher than 50 %, while can not get the data of $LD_{50.}$ The reason for explaining that it was PRGs in medium, *m*T was a high performance for seed germination on *Stylosanthes hamata* cv. Verano. The other studies were supported in high performance of *m*T which can help the seed growing although there were got the high radiation doses. Pdungsil *et al*. (2015) reported the various radiation doses of 0, 50, 100, 100 and 200 Gy. The results showed that the survival percentages for 30 days after irradiation were higher than 50 % in all treatments. Letham (1971) reported to promote radish cotyledons using cytokinin and explained by the promotion of cell enlargement, *m*T as a most active aromatic cytokinin as reported by Strnad *et al.* (1997) and Chiancone *et al*. (2016) studied of the effect on caulogenesis by *m*T and BA, micro-cuttings were cultured with 3 concentrations of each types of cytokinin on *Citrus tristeza virus*. The *m*T showed the result better than BA, use a low concentration can be giving a comparable multiplication rate. Structure of *m*T is similar to BA, it has not been associated with hyperhydricity, heterogeneity of plant growth or rooting suppression (Amoo *et al*., 2011; Bairu *et al*., 2007). In future should study this part to find the answer of LD_{50} using the media without PGRs, taken the higher radiation doses and culture.

It concluded that seeds of *Stylosanthes hamata* cv. Verano after 30 days cultivation found that 30 Krad decreased the percentage of shoot and root length of 31.7% and 20.51% respectively. The radiation dose was reduced $GR_{50(30)}$ at 39.82 Krad. $LD_{50(30)}$ at 26.14 Krad. The higher irradiation level increased the death rate. Seeds with irradiation on MS medium with 3 mg/L of *mT* after 60 days cultivation, the LD_{50} was higher than 50 % in all treatments and GR_{50} higher than 50 %. In the future, the improvement of Hamata bean would possible encounter the new characteristic by gamma irradiation in combination with PGRs to improve the growth rate.

Acknowledgement

The rhizome peanut *Stylosanthes hamata* cv.Verano. Hamata was provided by Feed and Forage Analysis Section. Animal Nutrition Division. Department of Livestock Development. Pathumthani Province, Thailand. Acknowledgements are due to Department of Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL).

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(Received: 20 November 2017, accepted: 30 December 2018)