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Application interval and concentration effect of gamma degraded chitosan on mulberry plant

*Mahmud, M., Talip, N., Yacob, N. and Idris, S.

Radiation Processing Technology Division, Malaysian Nuclear Agency, Bangi, 43000 Kajang, Selangor, Malaysia

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Abstract

Gamma radiation is one of the established tools to induce chain-scission of chitosan for low molecular weight chitosan preparation. The introduction of various radiation doses to chitosan powder produces degraded chitosan with various molecular ranges. They indicate different physicochemical and biological properties which are very important in product development. This study aimed to produce low molecular weight chitosan with an average molecular weight below 20 000 Da by exposing chitosan powder to gamma radiation at doses of 25, 50, 75 and 100 kGy. Then, to observe its growth-promoting activity on mulberry plants. The gamma-degraded chitosan was characterized by size exclusion chromatography-multi-angles laser light scattering (SEC-MALLS) and Fourier-Transform infrared (FTIR) to determine its weight-average molecular weight and molecular composition changes, respectively. After that, the gamma-degraded chitosan powder was dissolved in an acidic solution before spraying into mulberry. In the field, the mulberry plants were treated with 40, 80 and 100 ppm of degraded chitosan by foliar application. Each treatment consisted of 5 replicates of 2- 3 months-old mulberry plants which were randomly picked from the nursery. The control plants were treated with commercial growth promoter at concentration of 100 ppm which was applied weekly according to the farmer's practice. The study was carried out for 4.5 months at 2 different application intervals which were every two- and four-weeks' time. The mulberry plants treated with 80 ppm and 100 ppm degraded chitosan every two- and four-weeks showed better results on an average number of leaves and fruit, respectively compared to control.

1. Introduction

Agriculture is an important industry for the survival of a successful nation. The good quality of food and sufficient food supply nurture the development of great people. The application of extra agricultural input such as a growth promoter or plant regulator to increase quality and yield is an alternative taken by the farmers and manufacturers. Nowadays farmers and agriculture product manufacturers are opting for safe and clean technology as well as agriculture inputs due to the demand from consumers. Chemical or natural-based growth promoters are commercially available in the market to fulfil the needs. Natural-based promoters are materials obtained from animals, plants, algae and microorganisms that exist naturally (Hernández-Carmona *et al*., 2013). Polysaccharides are macromolecules composed of sugar units covalently chained by glycosidic bonds that are common compounds used for the development of natural-based growth promoters. It can be extracted from animals, plants, seaweed and fungi (Torres *et al*., 2019; Donato *et al*., 2021). Polysaccharide-based growth promoters such as low molecular weight chitosan, low molecular weight carrageenan and sodium alginate are proven to stimulate growth and enhance plant defence response system resulting in boosting plant productivity and protection from pathogenic microorganisms (Mondal *et al*., 2012; Mondal *et al*., 2013; Shukla *et al*., 2016).

Low molecular weight chitosan (LMCt) possesses plant growth promotion activity and is prepared through the degradation of chitosan. The chitosan can be degraded by chemical reaction (acidolysis, oxidative degradation), biological reaction (enzymatic hydrolysis) and physical reaction (radiolysis by gamma, microwave, electron beam and sonication) (García *et al*., 2015; Rokhati *et al*., 2018). Degradation of chitosan through acidolysis requires a high concentration of acid and a certain reaction temperature leading to the disposal of

*Corresponding author.
Email: *maznah@nm.gov.my*

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toxic waste. Meanwhile enzymatic hydrolysis demands specific enzymes and reaction conditions to degrade chitosan. Although it is considered safe and mild reaction, it produces low yield in long reaction time due to low reaction rate. The microwave, high energy electron beam (EB) and gamma-ray are physical methods to induce chitosan degradation with a high degradation rate, efficient process and low consumption of energy to produce degraded chitosan or LMCt (Rokhati *et al*., 2018). However, the drawback of microwave is, that it elevates the material temperature resulting in the deterioration of material. Therefore, it is suitable for certain samples only while EB has low penetration energy. Gamma rays seem the best alternative to degrade chitosan because the reaction can be done at any reaction temperature, is compatible with any state and size of materials and produces high purity of yield (Pandit *et al*., 2021).

Mulberry (*Morus* spp.) is a woody plant belonging to Moraceae family that grows and easily adapted to a wide range of climates; ranging from hot tropical, sub-tropical, temperate and arid climates (Rohela *et al*., 2020; Che Ariffin and Ismail, 2021). It can be planted in different forms of land including highlands, valleys and in harsh conditions of humid and semi-arid lands. Mulberry is planted for its leaf, stem, bark, fruits and roots for numerous purposes (Rohela *et al*., 2020). The barks and leaves are among the cheap resources of minerals, protein and crude fiber for ruminant feed and silkworms (Che Ariffin and Ismail, 2021). According to Saikim *et al*. (2017), 78667 ha lands in China were cultivated with mulberry for the cocoon industry through silkworm. In addition, mulberry fruits are processed for their natural colorant i.e. anthocyanins which is commercially used in foods, medicines and cosmetics (Kuan *et al*., 2020). Antioxidants such as carotene, vitamin C, Vitamin B1 and B2 also exist naturally in mulberry fruits. These antioxidants are used in pharmaceutical and cosmetic products (Che Ariffin and Ismail, 2021). Therefore, the mulberry plant is considered a multipurpose plant yet kind of low-cost and fast-growing plant.

This study was conducted to observe the effect of growth promotion activity and to determine the best treatment of low molecular weight chitosan (LMCt) on mulberry plants. The LMCt is a chitosan-based growth promoter and was prepared through gamma radiation-induced degradation of chitosan. The experiment played around with various LMCt dosages as well as application interval effects on mulberry plants.

2. Materials and methods

The industrial-grade chitosan powder with degree of deacetylation of >80% was purchased from a local supplier and its initial molecular weight and moisture content were determined. All other chemicals for sample preparation and analysis work were purchased from Corbion, Fisher and Sigma and used as received without any purification and modification.

2.1 Preparation of low molecular weight chitosan

Chitosan powder with an average of 10% moisture content was packed in polyethylene bag prior to irradiation. The irradiation was carried out at doses of 25, 50, 75 and 100 kGy with a dose rate of 1.62 kGy/ hour at MINTEC-Sinagama, Bangi, Selangor. The gamma radiation source is Cobalt-60. Technically, the chitosan powder was irradiated in a research loop which each of the complete irradiation cycles, the exposure dose was at 25 kGy. Then the irradiated chitosan powder will continue the same irradiation procedure to complete the requisite radiation doses which were 50, 75 and 100 kGy.

2.2 Characterization of gamma-irradiated chitosan

2.2.1 Molecular weight

The molecular weight of chitosan and irradiated chitosan were determined using size exclusion chromatography-multi angle laser light scattering (SEC-MALLS) equipped with Shodex 805 column from WatersTM, ultraviolet (UV) and refractive index (RI) detectors. The irradiated chitosan powder was dried in a vacuum oven at 40°C. Then, the irradiated chitosan powder was dissolved in 0.2 M acetic acid/0.1 M sodium acetate solution at a concentration of 2 g/L (Tahtat *et al*., 2012). The same 0.2 M acetic acid/0.1 M sodium acetate solution was used as the eluent during measurement. The measurement was done at 25°C with a flow rate of 0.75 ml/min.

2.2.2 Fourier-transform infrared spectroscopy

The Fourier transform infrared (FTIR) spectra were recorded on Bruker TENSOR FTIR spectrometer. The spectra were recorded with sums of 32 scans at resolution 4 cm^{-1} with a range of 400 – 5000 cm^{-1}. The chitosan and irradiated chitosan powder were dried prior to evaluation.

2.3 Pot test: application of low molecular weight chitosan on mulberry

The irradiated chitosan powder with average Mw 10 000 Da (below 20 000 Da) was dissolved in lactic acid to prepare LMCt solution. The pH of the LMCt solution was adjusted to pH 5.0 – 6.0 by 2 M sodium hydroxide. Then the LMCt stock solution was diluted with water into 3 different concentrations which were 40, 80 and

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100 ppm. Meanwhile, for commercial PGP, the solution prepared was according to the instructions stated on the bottle label by the manufacturer. The growth promoters were sprayed by hand sprayer in the morning.

Each treatment consisted of 5 pots of 2-3 months-old mulberry plant. For LMCt treatment, all samples were picked from the nursery which were not treated with any growth promoter. The stem cuts were soaked in water for 24 hours before being planted in a pot whereas, for control, the stem cuts were treated with growth promoter. All samples were irrigated according to the farmer's practice. The spraying schedule is indicated in Table 1.

Table 1. Application of PGP on mulberry plants.

Treatment	Application interval
Control	1 x every week
40 ppm LMCt, A40 80 ppm LMCt, A80 100 ppm LMCt, A100	1 x 2 weeks
40 ppm LMCt, B40 80 ppm LMCt, B80 100 ppm LMCt, B100	1 x 4 weeks

As for the application, the growth promoter was sprayed directly onto the leaves (up and underside) and stems in the morning. Due to the water-soluble property of the growth promoter, applying during rainy days was avoided and spraying was done on the following day, instead. The number of leaf and fruit were recorded in this study.

3. Results and discussion

The introduction of gamma radiation to polymer in a dry state induces polymer degradation through a chain scission reaction. Figure 1 indicates chitosan with an initial molecular weight (Mw) of 134.25 kDa decreased to 93.36 kDa, 42.50 kDa, 13.56 kDa and 10.10 kDa after irradiated at doses of 25, 50, 75 and 100 kGy, respectively. The reduction of chitosan molecular weight is due to chain scission of glycosidic bonds caused by gamma radiation (Kim *et al*., 2013; García *et al*., 2015). It also shows that the average Mw of chitosan decreased significantly after irradiation at 25 kGy and 50 kGy and the degradation rate became lower after irradiation at 75 kGy and 100 kGy. According to Navarro *et al*. (2018), chitosan exists as a polysaccharide with a higher proportion of amorphous region than crystalline. The shorter chain chitosan produced by radiation-chain scission has caused an increment in its crystallinity. The increment of crystallinity causes a decrease in its degradation rate. Therefore, even the high dose does not affect significantly on shorter chain chitosan. Besides the crystallinity factor, the degradation rate of chitosan by gamma radiation is also influenced by moisture content, air, oxygen presence, dose rate, inert gas presence and reaction temperature. Irradiation of chitosan in high content of moisture generates hydroxyl radicals from the radiolysis of water molecules that are involved in the chitosan chain degradation reaction (Tahtat *et al*., 2012).

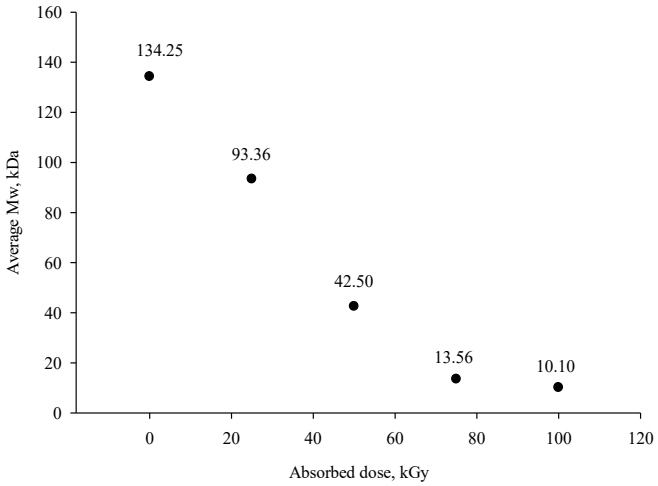

Figure 1. The reduction of average molecular weight of chitosan as a function of increasing irradiation dose.

Theoretically, gamma radiation is an ionizing radiation which is able to have a direct effect on the chitosan molecules whenever the irradiation is performed in dry and solid state (Tahtat *et al*., 2012). Figure 2 illustrates the chain-scission mechanism of the chitosan glycosidic bond induced by gamma radiation. The gamma radiation induces the hydrogen abstraction leading to the formation of unstable chitosan-macroradicals. The direct consequence of the interaction

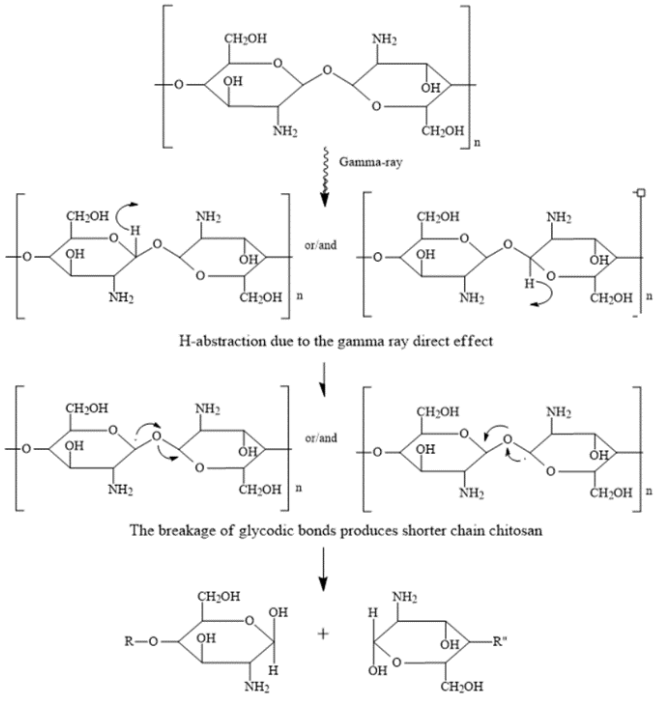

Figure 2. Degradation of chitosan due to cleavage of glycosidic bond induced by gamma radiation, where R and R" the repetition unit of D-glucosamine and N-acetyl-D-glucosamine (Kim *et al*., 2013; Navarro *et al*., 2018; Pandit *et al*., 2021).

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between chitosan and gamma radiation is chain scission reaction causes the breakage of glycosidic bonds leading to the formation of chitosan with shorter chain or described as degraded chitosan or chitosan oligomers (Orozco *et al*., 2012). From the result, irradiated chitosan with average Mw 13.56 kDa and 10.10 kDa are potential for the application as growth promoter (Duy *et al*., 2011; Dzung *et al*., 2017).

Figure 3 indicates IR spectra of chitosan powder and irradiated chitosan powder at doses of 25, 50, 75 and 100 kGy. The peaks of 3100–3600 cm^{-1}, 2800–2900 cm^{-1}, 1640 cm^{-1}, and 1100 cm^{-1} bands correspond to the overlapped N-H stretching vibration and O-H, C-H stretching vibration, N-H bending vibration and C-N of chitosan, respectively (Kim *et al*., 2013). The peak around 1020 cm^{-1} is attributed to C-O-C. The introduction of gamma radiation on chitosan increased the intensity of the peak at 3100–3600 cm^{-1} band. Meanwhile, the intensity of peak 1020 cm^{-1} corresponds to C-O-C decreased gradually. These situations indicate that the cleavage of glycosidic bonds (C-O-C) at β (1-4) occurred leading to the production of shorter-chain chitosan with lower crystalline phase content (Chmielewski 2010). The formation of shorter chain chitosan contributes to the increment of O-H intensity at 3100–3600 cm^{-1} band. Gamma irradiation indicates an insignificant effect at the N-H when observed at N-H bending vibration around 1648 cm^{-1}.

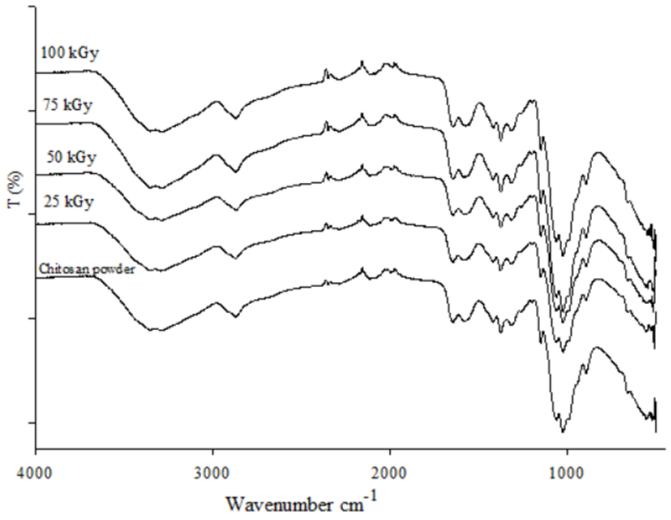

Figure 3. FTIR spectra of chitosan powder and irradiated chitosan powder at doses of 25, 50, 75 and 100 kGy.

Figures 4 (A) and (B) show the average number of leaves and fruit per plant after being treated with commercial PGP (Control) and LMCt at concentrations of 40, 80 and 100 ppm. The LMCt was applied every two weeks while commercial PGP was applied every week according to the farmer's practice. From week 2, mulberry plants treated with commercial PGP show higher leaf numbers compared to mulberry plants treated with LMCt at all concentrations. Only at week 14 onwards, treatment of LMCt started to demonstrate effect on mulberry plants. The average number of leaves per plant is higher after week 14 compared to Control, but the concentrations of LMCt indicate insignificant results of average leaf number per plant. On the other hand, Figure 4(B) displays plants treated with LMCt have higher average fruit number compared to Control. The LMCt treatment shows significant performance in fruit production from week 6 onwards. Figures 4(A) and 4(B) indicate that the treatment of LMCt every 2 weeks on mulberry plants enhances plant growth in fruit production but not on leaves number. The 80 ppm LMCt treatment is the recommended application dosage. According to Mondal *et al*. (2012) application of chitosan enhances the efficiency of fertilizer intake, increases the enzymatic nitrogen metabolism, and improves the transportation of nitrogen in functional leaves to boost plant productivity.

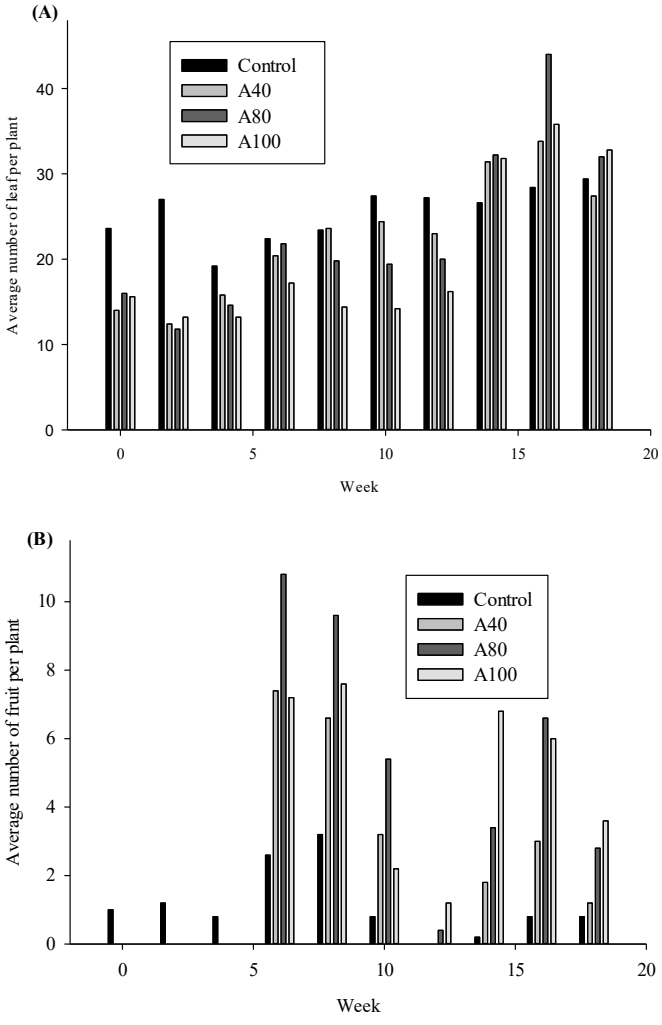

Figure 4. Foliar application of LMCt at 40, 80 and 100 ppm at the 2nd-week interval compared to commercial PGP, effect on mulberry plants.

Meanwhile, Figure 5(A) and (B) display the average number of leaves and fruit after being treated with LMCt at 40, 80 and 100 ppm every 4 weeks, respectively

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compared to Control. Figure 5 (A) indicates that treatment of LMCt at doses of 80 and 100 ppm resulted in better and similar performance on leaf development as control. The presence of amino groups in chitosan or LMCt might play a role in chlorophyll production and increase the number of chloroplasts per cell (Muley *et al*., 2019). The 40 ppm LMCt drops the average leaf number of mulberry plants. At the same time, the application of LMCt at the 4th-week interval shows a better effect on fruit production. Hussain *et al*. (2019) recorded that treatment of 90 ppm chitosan at the 25th-day interval increased plant growth and tomato quality because chitosan increases plant water uptake and enhances plant biochemical activity. Chitosan improves the N, P, and K uptake by plants. As the P increased, the photosynthesis activity increased leading to better plant productivity (fruit). At weeks 8 and 12, the production of fruits was highest after being treated with 80 ppm LMCt. However, the average fruit production at weeks 16 and 18 was inconsistent with yield in weeks 8 and 12.

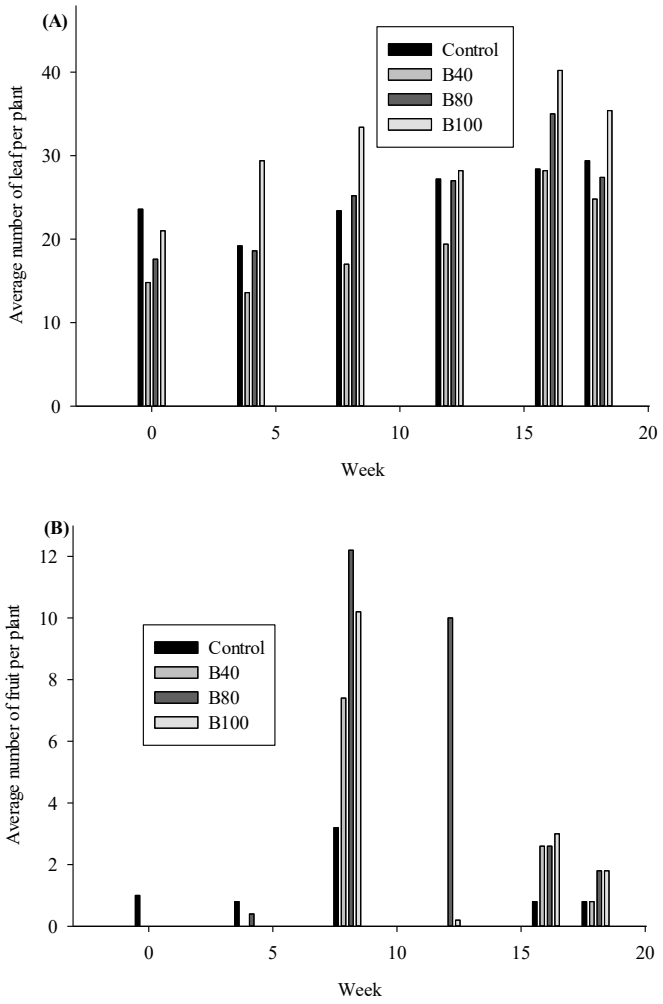

Figure 5. Foliar application of LMCt at 40, 80 and 100 ppm at the 4th-week interval compared to commercial PGP, effect on mulberry plants.

Figures 6 (A) and (B) show the effect of LMCt treatment on mulberry plants at application intervals of 2 weeks and 4 weeks. In Figure 6 (A) at 40 ppm LMCt average number of leaves is higher when applied at the 2 weeks interval compared to every 4 weeks. But the difference is insignificant. However, for 80 ppm and 100 ppm LMCt, the average number of leaves was higher after applied every 4 weeks compared to every 2 weeks. Application of LMCt at the 4 weeks interval at 80 ppm and 100 ppm is sufficient to induce growth promotion in mulberry plants in the aspect of leaf number. On the other hand, treatment of A80 and B80 show the highest average fruit number at weeks 6 and 8, respectively in Figure 6 (B). However, the fruit number started to reduce at week 10 for both treatments but the reduction of the A80 was greater than B80. Treatments of A40, B40, A100 and B100 indicate insignificant number of fruit production.

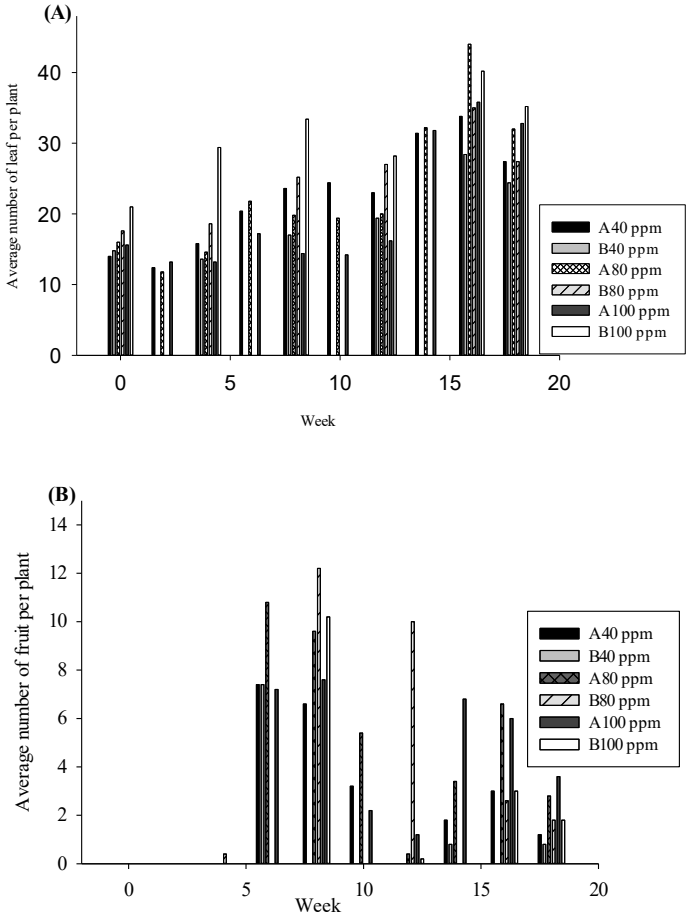

Figure 6. Effect of application intervals, every two weeks and every four weeks and LMCt concentration on growth of mulberry plants.

4. Conclusion

In this study, the LMCt can be prepared by gamma radiation-induced degradation of chitosan. The LMCt with Mw of 13.56 kDa prepared by 75 kGy of gamma exposure indicated plant growth promotion activity. The high irradiation doses induced chitosan chain breakdown but no indication of any changes in the main glucose ring structure. As for growth promotion activity, LMCt demonstrated similar results as commercial PGP

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although its application was less compared to commercial PGP. The application of LMCt at concentrations of 80 ppm and 100 ppm shows a great effect on the production of average leaves number and fruit number per plant, respectively. For the application interval once every 4 weeks treatment is sufficient to induce plant growth and increase yield production.

Conflict of interest

The authors declare no conflict of interest.

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