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# Research Article
# Mass Propagation of *Urena lobata* L. Hairy Root Possessing α-Glucosidase Inhibitory Activity by Using Suitable Culture Conditions

[1]Vu Thi Bach Phuong, [1]Cao Minh Dai, [1]Bui Lan Anh, [2]Pham Thi Anh Hong and [1]Quach Ngo Diem Phuong

[1]Department of Plant Biotechnology and Biotransformation, Vietnam National University of Ho Chi Minh City, Ho Chi Minh City, Vietnam
[2]Department of Biochemistry, Vietnam National University of Ho Chi Minh City, Ho Chi Minh City, Vietnam

## Abstract

**Background and Objective:** *Urena lobata* L., a member of the Malvaceae family, is commonly used as a traditional medicine in tropical and subtropical regions. The leaf and root extracts of *Urena lobata* L. have been used to treat several diseases such as diabetes, rheumatism, gonorrhea and toothache. This study aims at improving culture conditions of *Urena lobata* L. hairy root for biomass and α-glucosidase inhibitory activity. **Materials and Methods:** *Urena lobata* L. hairy roots were induced by infecting the *in vitro* leaf explants with *Agrobacterium rhizogenes* ATCC 15834 and were grown in WPM liquid medium under darkness. Several factors affecting the growth of *Urena lobata* L. hairy roots including of inoculum sizes (0.05, 0.1, 0.15 and 0.2 g/30 mL FW), concentrations of sucrose (2, 3, 4 and 5%) and macroelements composition (half, full, double and triple-strength WPM macroelements) were investigated. The combitation of improved factors were compared with the original process for biomass and the inhibition of α-glucosidase. **Results:** The combitation of improved factors using WPM medium with double-strength of macroelements composition supplemented with 4% (w/v) sucrose, an inoculum size of 0.1 g/30 mL FW has higher biomass of hairy root (1.33 times) than the original process. In addition, the hairy root cultivated in combination culture has maintained α-glucosidase inhibitory activity (during 40 days) longer than in individually improved factors and original process. **Conclusion:** A protocol for *Urena lobata* L. hairy root culture was established which could produce a material have longer α-glucosidase inhibitory activity.

Key words: *Agrobacterium rhizogenes*, hairy root culture, α-glucosidase inhibitor activity, *Urena lobata* L.

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Corresponding Author: Quach Ngo Diem Phuong, Department of Plant Biotechnology and Biotransformation, Vietnam National University of Ho Chi Minh City, Ho Chi Minh City, Vietnam



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

*Urena lobata* L., belonging to Malvaceae family, grows widely in the tropical and subtropical countries such as Bangladesh, China, Cuba, India, Indonesia, Malaysia, Nepal, Nigeria, Philippines and Vietnam. In these countries, this plant is used as a traditional herb to treat diverse ailments such as diabetes, colic, boils, pneumonia, cough, malaria, gonorrhea, fever, wounds, toothache, skin diseases and rheumatism[1-4]. A number of studies have been published that the extracts from *Urena lobata* L. leaves, roots and stems exhibit a variety of bioactivities such as anti-oxidant, anti-inflammatory, anti-microbial, anti-diarrheal, anti-diabetic, anti-hyperlipidemic and anti-diarrhoeal activities[4,5]. Moreover, recent studies have reported that the *Urena lobata* L. root extracts could reduce the blood glucose level in diabetic rabit[6] and the anti-diabetic potential of *Urena lobata* L. extracts could be mediated via the inhibition of dipeptidyl[7] peptidase IV. Since the promising bioactivities of *Urena lobata* L. extracts, several phytochemical studies have analyzed and identified different compounds from these extracts such as alkaloids, flavonoids, tannins, saponins, coumarins, steroids/triterpenoids, furocoumarins, mangiferin, quercetin, imperatorin, β-sitosterol, kaempferol, luteolin, hypolatin, gossypetin and stigmasterol[8-10].

In some Asian countries, the raw materials for herbal extracts can be collected from those plants grown on the farms or natural lands. The harvest of the raw materials from the wild is facing to several problems like misidentification, genetic and phenotypical variability and contaminations, especially in the case of root collecting. Hence, the cultivating of the medicinal plants in a well-controlled environment can help to overcome these difficulties. Along with other culture methods, the *in vitro* hairy root culture is also studied and applied to different medicinal plants, for examples, the hairy root culture of Buckwheat (*Fagopyrum esculentum* M.) to produce rutin[11] and the hairy root culture of *Atropa belladonna* using a bioreactor to increase the scopolamine content[12]. The finding of hairy root which is generated by the infecting of *Agrobacterium rhizogenes* to the explants has facilitated the study and application of this particular plant tissue in the plant secondary metabolites research and production[13]. In fact, the hairy root system has been used for studying plant secondary metabolites biosynthesis pathways, for production of valuable compounds or proteins from plants and even some current evidences indicated that this system can be applied for phytoremediation. The advantages of hairy root culture which can help to overcome some difficulties of *in vitro* plant tissue cultures include fast growing without adding of exogenous plant hormones, low doubling time, easy for maintenance, the ability to biosynthesize a range of compounds and to produce the similar metabolites to those of the mother plant or even the new secondary metabolites undetected in the mother plant nor in other kinds of *in vitro* tissues[14,15].

According to previous research published, *Urena lobata* L. hairy roots were a potential pharmaceutical source for the treatment of type 2 diabetes[16]. To improve culture conditions of *Urena lobata* L. hairy root for biomass and α-glucosidase inhibitory activity, the aim of this study focus on examine some factors affecting the growth of *Urena lobata* L. hairy roots in order to obtain high yield of quality hairy roots.

## MATERIALS AND METHODS

This study was implemented from May, 2017-November, 2018, in the Laboratory of Plant Biotechnology, Department of Plant Biotechnology and Biotransformation, Faculty of Biology and Biotechnology, Ho Chi Minh University of Science, Vietnam.

**Plant materials and growth conditions:** *Urena lobata* L. seeds were surface sterilized and placed on the MS basal medium[17] supplemented with 3% (w/v) sucrose and 0.8% (w/v) phytoagar (pH 5.8). The seeds were germinated in a growth chamber at 25±2°C under standard cool white fluorescent tubes with a 16/8 h photoperiod.

**Preparation of *Agrobacterium rhizogenes*:** *Agrobacterium rhizogenes* ATCC 15834 was obtained from RIKEN bank (Japan) through the MEXT project. *Agrobacterium rhizogenes* cells were grown in a yeast mannitol broth (YMB) medium in a shaking incubator (110 rpm, 25±1°C). Bacterial culture with approximate optical density (0.6±0.01) at 600 nm was used for infecting leaf explants of *U. lobata*.

**Hairy root culture conditions of original process:** Leaves of 15 days old *in vitro* seedlings of *Urena lobata* were used for induction of hairy roots by *Agrobacterium rhizogenes* ATCC[16] 15834. *Urena lobata* hairy roots were grown in 250 mL shake flasks, containing 30 mL of liquid WPM medium supplemented with 3% sucrose and an inoculum size of 0.1 g/30 mL on a rotary shaker with 80 rpm at 25°C under darkness.

**Influence of some factors on culture conditions:** Except for the factors investigated, the remaining factors were fixed and retained as in the original process of hairy roots culture conditions. The hairy roots were harvested and determined the dry weight (DW) and the activity of α-glucosidase inhibition every 5 days of the 40-day culture.

**Investigation of inoculum sizes:** The input amounts of hairy roots were tested including 0.05, 0.1, 0.15 and 0.2 g/30 mL (1.67, 3.33, 5.00 and 6.67 g $L^{-1}$).

**Investigation of sucrose concentrations:** Different sucrose concentrations were supplemented in medium culture including 2, 3, 4 and 5% (w/v).

**Investigation of macroelements composition in WPM medium:** The hairy roots were grown in liquid WPM (Woody Plant Medium) medium[18] with half, full, double and triple-strength of macroelements composition (symbol ½WPM, WPM, 2WPM, 3WPM, respectively).

**Investigation of the combined effect of improved factors on hairy root culture conditions:** The hairy roots are cultured in two processes: The original process and the improvement process for hairy root culture conditions (inoculum sizes, sucrose concentrations and macroelements composition in WPM medium).

**α-glucosidase inhibitory activity:** The α-glucosidase inhibitory activity was performed as previous described[19] with some modifications. Powder of hairy roots (1 g) was extracted with 100 mL ethanol on an orbital shaker for 24 h to make sample solution. The α-glucosidase solution (0.2 U $mL^{-1}$) and 5 mM p-nitrophenyl-α-D-glucopyranoside (pNPG) solution were prepared in 0.1 M phosphate buffer (pH 6.8). The mixture of 50 µL of sample solution and 40 µL of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U mL) was incubated at 37°C for 20 min and then added 40 µL of 5 mM pNPG solution. The reaction was happened for 20 min at 37°C and terminated by adding of 130 µL of 0.2 M $Na_2CO_3$. The control had 50 µL of buffer solution in place of the extract of hairy roots. In the blank sample, enzyme solution was replaced by buffer solution. Acarbose was used as a positive reference. The reaction mixture was measured at 405 nm. The α-glucosidase inhibitory activity was shown as percentage inhibition which was calculated using follow equation:

$$\text{Inhibition (\%)} = \frac{\begin{array}{c}(\text{Blank control absorbance} - \text{Control absorbance}) - \\ (\text{Blank sample absorbance} - \text{Sample absorbance})\end{array}}{\text{Blank control absorbance} - \text{Control absorbance}} \times 100$$

**Statistical analysis:** Each treatment included 10 erlenmeyer flasks in replicates of three. The inhibition of α-glucosidase activity (%) and the dry weight (DW) of hairy root biomass were scrutinized after each 5 days of the 40 day culture. All data analysis were performed using the SPSS 16.0 (Copyright SPSS Inc.). Experimental results were represented as mean±standard deviation (SD). Differences between means were evaluated by Duncan's multiple range tests. Statistical significance was accepted at a level of $p<0.05$.

## RESULTS AND DISCUSSION

**Effect of inoculum size on hairy root growth:** The influence of inoculum size (from 0.05-0.2 g/30 mL FW) on *Urena lobata* L. hairy root growth was examined (Fig. 1). As shown in Fig. 1, the 0.1 g/30 mL FW inoculum size did not trigger the growth of hairy roots as rapid as the 0.15 and 0.2 g/30 mL in the stationary phase (5th-20th day), however, the hairy roots from all of inoculum sizes reached to a similar dry weight on the 30th day (approximately 0.5 g DW). Regarding α-glucosidase inhibitory activity, on the 30th of culture, only the activity of 0.1 g/30 mL FW inoculum size (99.18%) was higher than the others and this activity was not significantly reduced in the following days. Thus, the 0.1 g/30 mL FW inoculum size seems to be more effective than the 0.15 and 0.2 g/30 mL due to the saving of input hairy roots amount while the output biomass did not show any significant differences.

**Effect of different concentrations of sucrose:** The hairy roots were grown in the liquid WPM medium supplemented with different concentrations of sucrose (2, 3, 4 and 5% (w/v)). While the hairy roots grew slowly in the media supplemented with low concentrations of sucrose (2 and 3%), the medium contained 4% of sucrose seemed to accelerate the growth of hairy roots and inhibition of α-glucosidase of 4% sucrose is also stronger than the other concentrations (Fig. 2). In 30th culture, the hairy roots were cultured in 4% sucrose medium reached to the highest biomass (0.73 g DW) which was almost 1.4 times higher than those grown in the initial condition (0.52 g DW). The dry weight of *U. lobata* hairy root increased with increase in sucrose concentration in the range of 2-4% because plant cells often break down sugars as a source of carbon for growth. The medium supplemented with 5% of sucrose did not support to the growth of hairy roots which looked unhealthy with the browner color than those growing on other conditions and inhibition of α-glucosidase is lower than the sucrose concentrations of 3 and 4% (Fig. 2).

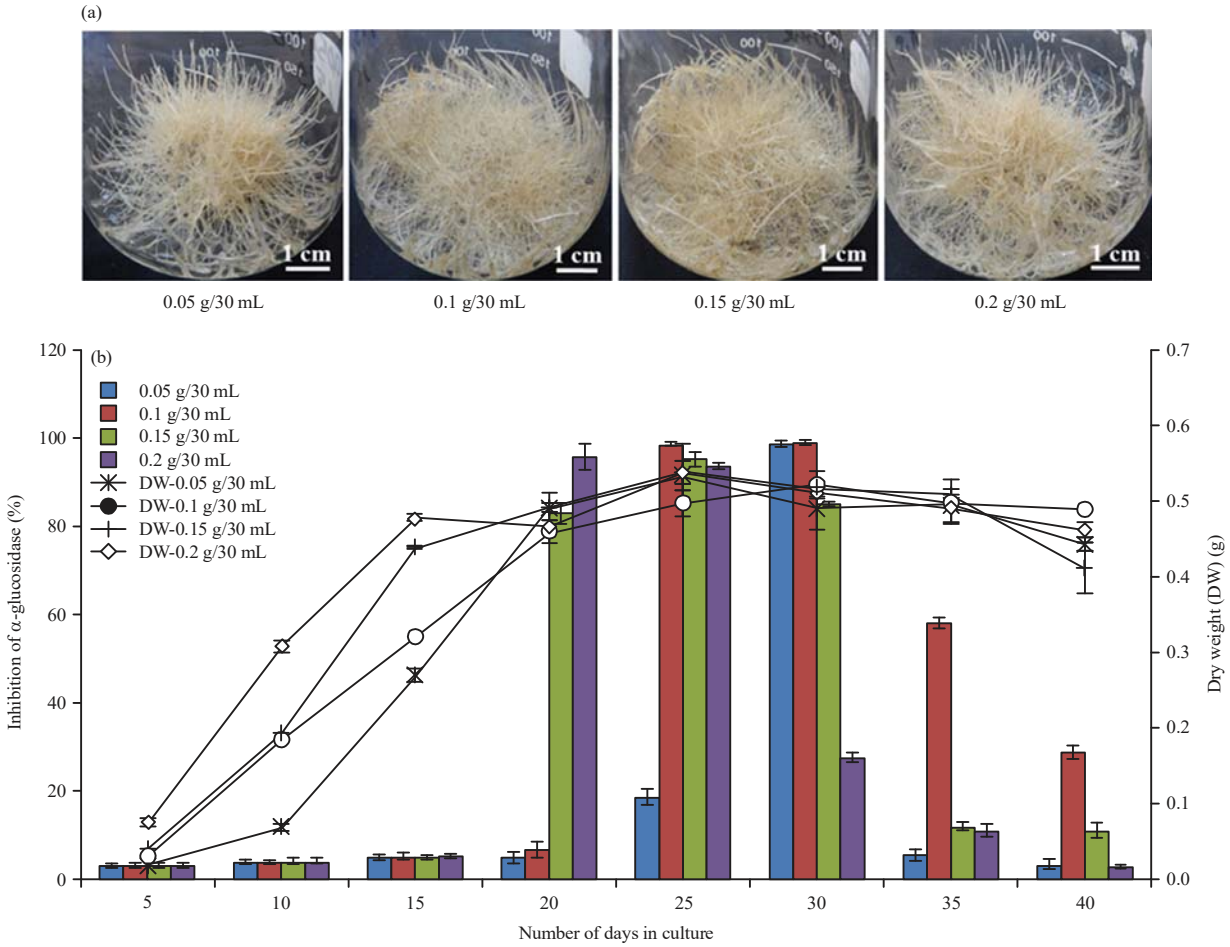


Fig. 1(a-b): Effect of inoculum size on *Urena lobata* L. hairy roots growing (25th day), (a) Dry weight and (b) α-glucosidase inhibition profile

**Effect of different macroelements composition in WPM medium:** In the present study, the growth of *Urena lobata* L. hairy roots was tested in WPM medium with different of macroelements composition such as half, full, double and triple-strength WPM (symbol 1/2 WPM, WPM, 2 WPM, 3 WPM, respectively). As shown in Fig. 3, the hairy roots grew slowly in the 1/2 WPM medium while hairy roots in the 2 WPM medium developed faster and inhibition of α-glucosidase was also higher than the others (Fig. 3). The hairy roots in 3 WPM medium obtained the highest fresh weigh on 25th day but dry weigh was similar to 2 WPM medium. In addition, the hairy roots growing in 3 WPM medium on the 30th day started to appear the browner color, looked unhealthy and showed signs of death on the 40th day. Furthermore, the inhibition of α-glucosidase of hairy roots in 3 WPM medium is lower than the hairy roots in WPM and 2 WPM medium. In summary, 2 WPM medium is more suitable for growth and α-glucosidase inhibitory activity of *Urena lobata* L. hairy roots than 1/2 WPM, WPM and 3 WPM mediums.

**Effect of the improved factors combination:** Eventually, the *Urena lobata* L. hairy roots were cultured under the improved conditions which were combined the most suitable conditions from above tests including 0.1 g/30 mL for the inoculum size, the double-strength of macroelements composition 2 WPM medium supplemented with 4% sucrose. As shown in Fig. 4, the growth of hairy roots under the improved conditions was notably faster and healthier than under initial conditions. On the 25th day, biomass of hairy root which is cultured in improved conditions is higher 1.33 times than the initial condition. The hairy roots grown under initial conditions reached to stable phase from the 25th-30th day and started decreasing the growth rate afterward while the improved conditions maintained both the high growth rate of hairy roots and inhibition of α-glucosidase up to 40 days of culture (Fig. 4).

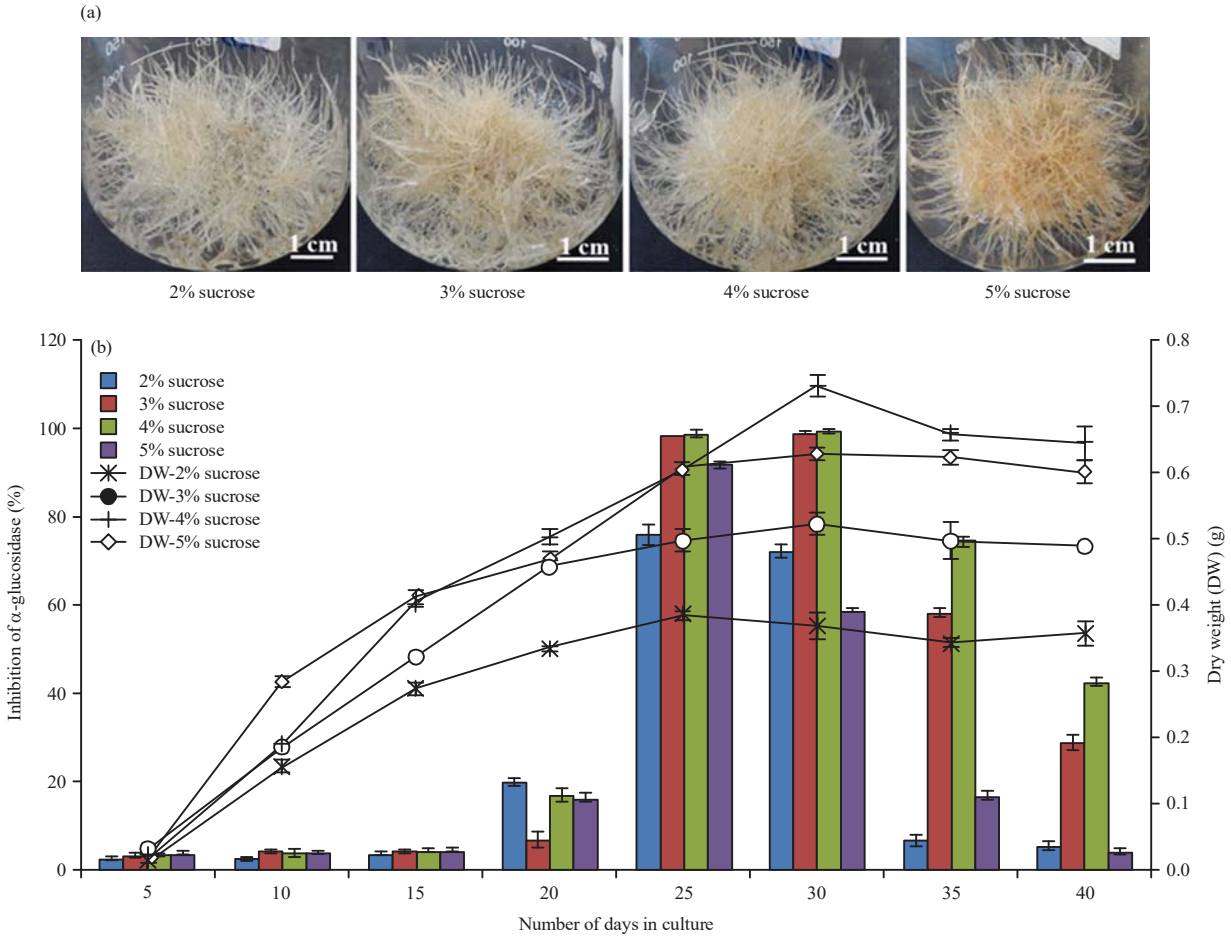


Fig. 2(a-b): Effect of sucrose concentrations on *Urena lobata* L. hairy roots growing (25th day), (a) Dry weight and (b) α-glucosidase inhibition profile

**Effect of inoculum size on hairy root growth:** It is well known that the input inoculum density is an important factor affecting the growth of hairy roots in shaking flasks[20]. The optimum inoculum size was found as a fundamental factor for the successful root culture in *Withania somnifera* (L.) Dunal[21]. The growth kinetics of *Atropa belladonna* hairy roots in a shaking flask were found to be varied depending on the inoculum size and initial medium volume[20]. When the plant tissues are cultured in the liquid medium, the suitable inoculum size is an important factor which can increase the metabolite production[22]. Taken together, these results indicate that the 0.1 g/30 mL FW is more appropriate for biomass production of *Urena lobata* L. hairy root as well as the optimum α-glucosidase inhibitory activity in the range of surveyed inoculum sizes.

**Effect of different concentrations of sucrose:** Carbohydrates, especially sucrose, are important carbon and energy sources for most of the plant cell lines. It has been demonstrated that the initial sucrose concentration can affect the growth rate and secondary metabolites production of cultured plant cells[23,24]. High concentration of sucrose can improve the biomass and lead to increase the production of secondary metabolite. However, over sucrose supplement may cause osmotic stress even plant cells death.

**Effect of different macroelements composition in WPM medium:** The nutrition of culture medium is also important for the growth of plant tissues and their secondary metabolites production[15]. In fact, previous studies had shown that the composition and concentration of the medium could have a significant impact on hairy root growth in the culture systems[25]. Low concentration of macroelements is not sufficient for the growth of hairy root and also decrease the bioactivity. Moreover, hairy root could be osmotic stress when cultured in extreme high macroelements.

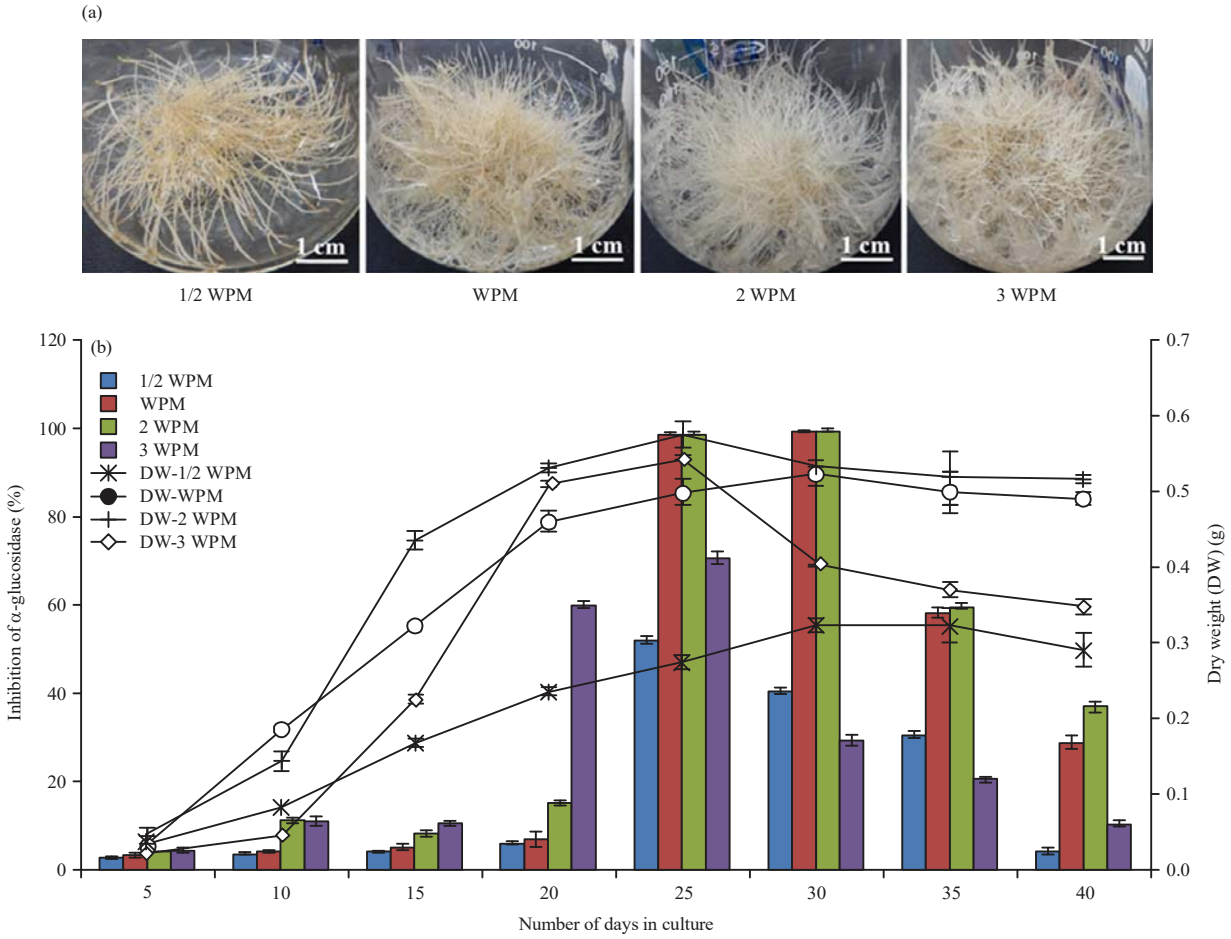


Fig. 3(a-b): Effect of different macroelements composition on *Urena lobata* L. hairy roots growing (25th day), (a) Dry weight and (b) α-glucosidase inhibition profile

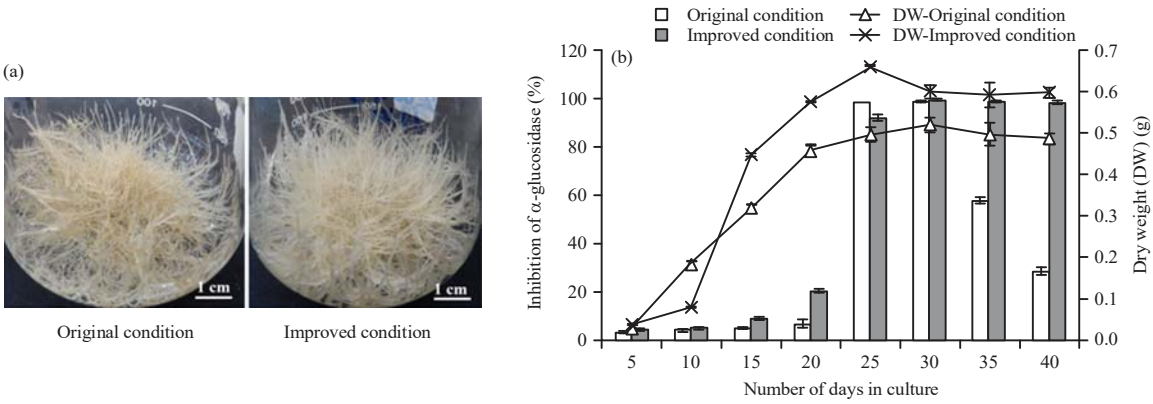


Fig. 4(a-b): Comparison of original with improved conditions on *Urena lobata* L. hairy roots growing (25th day), (a) Dry weight and (b) α-glucosidase inhibition profile

**Effect of the improved factors combination:** Improved culture conditions will increase root biomass with α-glucosidase inhibition ability more stable than in individually improved factors and in original culture condition. The advantage of this combination is maintain the activity of bioactive compounds in cultured hairy roots even in lag phase.

## CONCLUSION

The *Urena lobata* L. hairy roots were cultured with suitable factors that can help to increase the biomass and inhibition of α-glucosidase activity. The suitable growth conditions were observed as follows: the 2 WPM liquid medium supplemented with 4% (w/v) sucrose and 0.1 g/30 mL of the inoculum size. These results have contributed to a whole protocol for *Urena lobata* L. hairy root culture which can be applied for inhibition of enzyme α-glucosidase to type 2 diabetes treatments.

## SIGNIFICANT STATEMENT

This study discovered the impact of suitable factors which may enhance the biomass accumulation and α-glucosidase inhibitory activity of *Urena lobata* hairy root. By improving and then putting together inoculum size, concentration of sucrose and macroelements factors in one cultural process that can be beneficial for developing growth and increasing bioactivity in culture of hairy root. This study will help the researchers to uncover the critical areas of biomass propagation of hairy root that many researchers were not able to explore. Thus a new theory on using α-glucosidase inhibitory activity for screening the created hairy root.

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