



Influences of Ammonium Nitrate on Growth and Inulin Content of Jerusalem Artichoke (*Helianthus tuberosus* L.) *in Vitro*

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Abstract

Jerusalem artichoke (*Helianthus tuberosus* L.) consists of high inulin and fructose, useful for the pharmaceuticals and chemical industries. Therefore, previous researchers have studied on the micropropagation of Jerusalem artichoke from seeds, nodes, and young leaves; effects of sugar and plant growth regulators on culture and inulin content in Jerusalem artichoke; and microtuber propagation. However, studies on the effects of ammonium nitrate (NH_4NO_3) on growth and biosynthesis of inulin in Jerusalem artichoke are scarce at present. Therefore, the objective of this work was to evaluate the effect of NH_4NO_3 on the growth of Jerusalem artichoke and its inulin content *in vitro*. Auxiliary meristems from the field-grown plants were cultured on MS medium containing 0, 825, 1,650, and 3,300 mg/L NH_4NO_3 . The inulin content of plantlets was analyzed by HPLC-RID after subculturing every 4 weeks for 5 times. The results showed that shoot length, node number, leaf number, leaf width, leaf length, fresh weight, and dry weight were maximum in 3,300 mg/L NH_4NO_3 . While shoot number and root number were best in 1,650 mg/L NH_4NO_3 . Furthermore, the study found maximum inulin content on MS containing 1,650 mg/L NH_4NO_3 and higher hyperhydricity in higher concentrations of NH_4NO_3 . Overall, MS containing 1,650 mg/L NH_4NO_3 showed a positive effect on vegetative growth, hyperhydricity, and inulin production of Jerusalem artichoke. It can be used as an alternative approach for propagation and inulin production from Jerusalem artichoke.

Keywords: Ammonium nitrate, HPLC – RID, Inulin, *In vitro*, Jerusalem artichoke

Introduction

Jerusalem artichoke (*Helianthus tuberosus* L.) belongs to the Asteraceae family. It is a native plant of North America, where it gradually spread to other countries, including Thailand (Stanley & Stephen, 2007). In Thailand, Jerusalem artichoke is known as Kaentawan. It can adapt to hot climates, thereby, improving growth rate, yields, and biomass. The cultivation and maintenance costs are desirable because it requires low inputs, including pesticides, fertilizers, and water. It consists of a high inulin and valuable source of fructose, which is useful for food, fodder, pharmaceuticals, and chemical industries (Kays & Nottingham, 2008). Inulin is a type of polysaccharide in the fructan group, usually found in the tubers and fruit, including dahlia, chicory, Jerusalem artichoke, onion, and banana (Suzuki, 1993). Inulin is used in diabetics because it has a positive effect on blood glucose lowering, lipid balance, mineral uptake, and immune regulation. Inulin also has the properties of prebiotic, which stimulates the growth and activity of bacteria that are needed in the large intestine, which will improve the health of the host. Jerusalem artichoke is also used in feed industries in term of silage production to stimulate growth and improve productions (Seiler, 1993; Gibson, Beatty, Wang, & Cummings, 1995; Coussement, 1999). In addition, it provides a substrate



for microorganisms in the fermentation process to produce acetone, butanol, and ethanol due to its high sugar content serving as alternative energy (Kaur & Gupta, 2002).

Pythium reduces the yield and quality of Jerusalem artichoke. Therefore, the plant tissue culture technique is applied to produce disease-free plantlets. Plant tissue culture plays an important role in the production of active compounds in plant metabolites such as alkaloids, anthocyanin, carotenoids, inulin, etc. Nitrogen is a component of protein, nucleic acid, and chlorophyll in plants. It significantly affects growth and secondary metabolite formation in plant tissue culture (George, Hall, & Klerk, 2008). Ammonium nitrate (NH_4NO_3) as a macronutrient plays an important role in plant growth and induces inulin *in vitro*. Following other studies, HPLC was used in this study to analyze inulin content, using cation-exchanged column, coupled with refractive index (RID) (Antošová, Polakovič, & Báleš, 1999; Petkova, Vrancheva, Denev, Ivanov, & Pavlov, 2013; Retnaningtyas, 2012). Currently, there are many studies relate to the micropropagation of Jerusalem artichoke from seeds, node, and young leaves; effects of sugar and plant growth regulators on culture and inulin content in Jerusalem artichoke; and micro-tuber propagation. However, the effects of ammonium nitrate on the growth and biosynthesis of inulin in Jerusalem artichoke are not studied deeply.

Therefore, the objective of this work was to evaluate the effect of NH_4NO_3 on the growth of Jerusalem artichoke and its inulin content *in vitro*. Findings of this study will be served as a guideline to produce disease-free and high inulin content plantlets to propagate and increase the productivity of Jerusalem artichoke.

Methods and Materials

Effect of NH_4NO_3 on vegetative growth of Jerusalem artichoke *in vitro*

Preparation of materials and experiments

Jerusalem artichoke cultivar “HEL 65” tubers were obtained from the varietal improvement of peanut and Jerusalem artichoke for increasing product value and quality as a functional food research group, Faculty of Agriculture, Khon Kaen University, Thailand. These were used as plant materials for this research. Tubers were washed with detergent and tap water and kept under dark conditions for 14 days for sprouting. The planted tubers were placed on a medium, consisting of raw husk, husk, coir, and soil at 1:1:1:1 for 15 days of sprouting. After 15 days of planting, the healthy nodes of Jerusalem artichoke were washed with soap and tap water. The nodes surface sterilization was soaked in 70% ethanol for 1 minute, then washed 3 times with sterile distilled water and soaked again in 3% sodium hypochlorite (NaOCl) of Clorox® solution with 2 drops of Tween 20 for 12 minutes. Subsequently, they were rinsed 3 times with sterile distilled water. Following Murashige and Skoog (1962), we aseptically cultured these nodes on MS medium containing sucrose (30 g/L), phytigel (2.4 g/L), and different concentrations of 0, 825, 1,650, and 3,300 mg/L NH_4NO_3 , added prior to the autoclave at 121 °C and 15 psi for 15 minutes. In this study, the pH of the medium was adjusted to 5.7. The cultivation was cultured in light conditions for 16 hours/day and subcultured every 4 weeks. Then, the harvested platelet was used for inulin extraction and the samples were analyzed of every subculture time.



Extraction and analysis of inulin contents using the HPLC-RID

Harvested plantlets were selected, freeze-dried at -52°C for 3 days and crushed. For extraction, the method from Gaafar, El-din, and Boudy (2010) was used, mixed dried explants with hot water at 80°C for 90 minutes with a ratio of 1:5. The extracted samples were analyzed for inulin by using HPLC according to the modified method from Petkova et al. (2013). HPLC from Shimadzu Company, Japan coupled with LC-20AD pump and RID-10A Detector, performed the chromatographic separation. The software program Class - VP was used to control the system, data acquisition, and data analysis. Analytical column Shodex[®] Sugar KS-806 (300 mm \times 8.0 mm i.d.) and sugar KS-G-6B guard column (50 mm \times 6.0 mm i.d.). The mobile phase was deionized water, vacuum-filtered through 0.45 μm membranes before using, and then prepared standard solutions using analytical graded reagents and solvents. Stock solutions of glucose, sucrose, fructose, kestose, nystose, and inulin with initial concentrations of 10 mg/mL were prepared with water (hot water just for inulin solution). A series of each standard solution containing 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, and 10 mg/mL were prepared from the stock standard solutions.

Data collection and statistical analysis

The data of shoot length (cm); node number (nodes/plantlet); leaf number (leaves/plantlet); root number (roots/plantlet); shoot number (shoots/plantlet); leaf width (cm); leaf length (cm); root length (cm); fresh weight (g/vessel); dry weight (g/vessel); the percentage of hyperhydricity (%); and inulin contents (g/100 g of dry weight) were collected. This study adopted completely randomized design (CRD) with 10 replicates. Data were analyzed by using One-Way Analysis of Variance (ANOVA) and compared the differences between groups by Least Significant Difference (LSD) in Statistix 8 program.

Results

Effect of NH_4NO_3 on vegetative growth of Jerusalem artichoke *in vitro*

Healthy nodes of Jerusalem artichoke (15 days) were cultivated on MS medium containing sucrose (30 g/L), phytigel (2.4 g/L), and different NH_4NO_3 concentrations (0, 825, 1,650, and 3,300 mg/L) for 4 weeks. The result showed that 3,300 mg/L NH_4NO_3 concentration resulted in the longest shoot length of 6.19 cm, followed by the concentrations of 1,650, 825, and 0 mg/L NH_4NO_3 with shoot length of 3.17, 2.88, and 2.13 cm, respectively. The highest leaf width was 0.93 cm in 3,300 mg/L NH_4NO_3 , followed by 0.65, 0.62, and 0.45 cm in 825, 1,650, and 0 mg/L NH_4NO_3 , respectively. The results also showed the longest leaf length of 2.32 cm in 3,300 mg/L NH_4NO_3 , followed by 1.45, 1.55, and 1.56 cm in 0, 825, and 1,650 mg/L NH_4NO_3 , respectively. Although there were no significant differences in fresh weight and dry weight, the results showed the highest fresh weight (1.19 g/vessel) and dry weight (0.15 g/vessel) in 3,300 mg/L NH_4NO_3 . Collectively, results indicated that the high concentrations of NH_4NO_3 contribute to the vegetative growth as shown in Table 1.

**Table 1** Effect of different NH_4NO_3 concentration on MS medium on shoot length, leaf width, leaf length, fresh weight, and dry weight of Jerusalem artichoke (*Helianthus tuberosus* L.) *in vitro* after 4 weeks cultured.

Concentration of NH_4NO_3 (mg/L)	Shoot length (cm)	Leaf width (cm)	Leaf length (cm)	Fresh weight (g/vessel)	Dry weight (g/vessel)
0	2.13 ^b	0.45 ^c	1.45 ^b	0.33	0.04
825	2.88 ^b	0.65 ^b	1.55 ^b	0.33	0.04
1,650	3.17 ^b	0.62 ^{bc}	1.56 ^b	0.32	0.03
3,300	6.19 ^a	0.93 ^a	2.32 ^a	1.19	0.15
P-value	*	*	*	ns	ns

Note: Data shown are the average values of vegetative growth in each NH_4NO_3 concentration. Within a column, mean values followed by different superscript letters differ significantly at $P < 0.05$ (LSD test); * = significant at $P < 0.05$; ns = non-significant.

Furthermore, the concentration of 3300 mg/L NH_4NO_3 resulted in the highest node number of 6.70 nodes/plantlet, followed by 825, 1650, and 0 mg/L NH_4NO_3 with 4.70, 4.50, and 2.95 nodes/plantlet, respectively, as presented in Table 2. The maximum leaf number was 14.20 leaves/plantlet in 3300 mg/L NH_4NO_3 . The result showed the highest shoot number of 2.8 shoots/plantlet in 1,650 mg/L NH_4NO_3 , followed by 2.40, 1.70, and 1.30 shoots/plantlet in 825, 0, and 3,300 mg/L NH_4NO_3 , respectively. Although the concentration of 1,650 mg/L NH_4NO_3 resulted in the highest root number (1.00 roots/plantlet) and the longest root length (3.68 cm), there were statistically no significant differences.

Table 2 Effect of different NH_4NO_3 concentration on MS medium on node number, leaf number, shoot number, root number, and root length of Jerusalem artichoke (*Helianthus tuberosus* L.) *in vitro* after 4 weeks cultured.

Concentration of NH_4NO_3 (mg/L)	Node number (nodes/plantlet)	Leaf number (leaves/plantlet)	Shoot number (shoots/plantlet)	Root number (roots/plantlet)	Root length (cm)
0	2.95 ^c	6.70 ^c	1.70 ^{ab}	0.50	1.92
825	4.70 ^b	10.20 ^b	2.40 ^{ab}	0.50	2.91
1,650	4.50 ^b	10.95 ^b	2.80 ^a	1.00	3.68
3,300	6.70 ^a	14.20 ^a	1.30 ^b	0.50	1.66
P-value	*	*	*	ns	ns

Note: Data shown are the average values of vegetative growth in each NH_4NO_3 concentration. Within a column, mean values followed by different superscript letters differ significantly at $P < 0.05$ (LSD test); * = significant at $P < 0.05$; ns = non-significant.

The results of the study also showed higher hyperhydricity when NH_4NO_3 concentrations were increased. The results in Table 3 showed 20% hyperhydricity in 1,650 mg/L NH_4NO_3 and 60% hyperhydricity in 3,300 mg/L NH_4NO_3 . The results were similar to previous work Modi, Sinha, and Kothari (2009) who studied the reduction of hyperhydricity in micro-propagated French marigold (*Tagetes patula* L.) plants by modifying the medium parameter in 0, 412.5, 825, 1,650, and 3,300 mg/L NH_4NO_3 on MS. + BA (1 mg/L) + IAA (0.5 mg/L). They reported that in 3,300 mg/L NH_4NO_3 on MS medium resulted in callus like-body in plantlet and occurred 100 % hyperhydricity.

**Table 3** Hyperhydric shoots of Jerusalem artichoke (*Helianthus tuberosus* L.) on MS medium after cultured for 4 weeks.

Concentration of NH_4NO_3 (mg/L)	Hyperhydricity (%)
0	0
825	0
1,650	20
3,300	60

Effect of NH_4NO_3 on inulin contents of Jerusalem artichoke *in vitro*

The stress response was monitored from different concentrations of NH_4NO_3 on inulin contents using the HPLC-RID. The result showed the induction potential of 0 mg/L NH_4NO_3 on inulin contents of Jerusalem artichoke *in vitro* as 3rd, 4th, and 5th subculture detected the inulin as well. However, the highest inulin content (3.07 g/100 g of dry weight) was detected at 1,650 mg/L NH_4NO_3 of 3rd subculture time as shown in Table 4. The subsequent cultures in 1,650 mg/L NH_4NO_3 did not detect any inulin content. In addition, the study did not detect inulin in 3300 mg/L NH_4NO_3 because vegetative growth has used NH_4NO_3 , as a result, there are no secondary metabolites (George et al., 2008). Wang and Tan (2002) also studied the artemisinin production from root cultures of *Artemisia annua* by varying the ratio of nitrate to ammonium ($\text{NO}_3^- : \text{NH}_4^+$). They reported that when the ratio of NH_4^+ was higher, the growth and the amount of artemisinin in the root culture of *Artemisia annua* decreases.

Table 4 Effect of NH_4NO_3 concentration in MS medium on inulin contents in water extracts obtained from Jerusalem artichoke (*Helianthus tuberosus* L.) *in vitro* using the HPLC-RID method.

Concentration of NH_4NO_3 (mg/L)	Inulin contents (g/100 g of dry weight)				
	Subculture times				
	1	2	3	4	5
0	N.D.	N.D.	1.61 ^c	1.43 ^d	2.73 ^b
825	N.D.	N.D.	N.D.	N.D.	0.88 ^f
1,650	N.D.	N.D.	3.07 ^a	N.D.	N.D.
3,300	N.D.	N.D.	N.D.	N.D.	N.D.

Note: Data shown are the average values of inulin content in each NH_4NO_3 concentration. Within a column, mean values followed by different superscript letters differ significantly at $P < 0.05$ (LSD test); N.D. = not detectable

Discussion

The results of this study were clearly indicated that NH_4NO_3 was an essential factor for the growth and inulin biosynthesis of Jerusalem artichoke *in vitro*. We found that high concentrations of NH_4NO_3 gave a positive effect on vegetative growth, while low concentration restricted the growth of plant organs due to nitrogen deficiencies. For instance, we observed abnormal symptoms in shoots, such as reduced growth, lower number of shoots, and the chlorotic appearance of leaves. Enhancements of NH_4NO_3 concentration were toxic due to high ammonium levels, causing negative effects on plant growth and survival (Ho & Tsay, 2010). Although high concentration gave a good vegetative growth, it also resulted in high hyperhydricity. The Symptoms of hyperhydric plants are translucent, reflecting the scarcity of chlorophyll and the high water content caused by thin or hard tissue layers, reduced number

of palisade cells, stomata abnormal, less developed cell walls, and the large cell spaces in the mesophyll cell layer (Kevers, Franck, Strasser, Dommès, & Gaspar, 2004; Rojas-Martínez, Visser, & de Klerk, 2010; Ivanova and Van Staden, 2010). The finding of this study agreed with a previous study (Kevers et al., 2004). They also noted that high ammonium concentration as the main cause of hyperhydricity *in vitro*. According to Saurabh and Kiran (2015), the use of lower cytokinin content combine with ammonium nitrate in the media and reduced the ratio between ammonium and nitrate in the medium can control hyperhydricity from high NH_4NO_3 .

The present investigation showed that an enhancement of the NH_4NO_3 concentration in the culture medium led to an increase of inulin contents in the plantlets. However, the excessive concentration of NH_4NO_3 reduced the accumulation of inulin contents in the plantlets due to hyperhydricity. This study has shown that the growth and inulin contents of the Jerusalem artichoke plantlets were inhibited at higher concentrations of NH_4NO_3 . In previous studies, secondary metabolite production of Asteraceae plants in flask cultures or bioreactors, MS medium characterized with higher nitrogen concentration has often been selected as the basal medium by most investigators (Qin, Li, Yun, Ye, & Li, 1994; Legha et al., 2012; Liu, Guo, Wang, & Ouyang, 2003). Similarly, this study found that the optimum concentration of ammonium nitrate for cultivation was 1,650 mg/L in both of vegetative growth and inulin production. This work is useful for further regulation and optimization of node cultures of Jerusalem artichoke for the efficient propagation and production of inulin on a large scale.

Considering the result in Figure 1 the characteristics of sample plantlets after being cultured on MS medium for 4 weeks at different NH_4NO_3 concentrations. It clearly shows a better vegetative growth of plantlet including long shoot, large leaf, more leaf, and more nodes in 3,300 mg/L NH_4NO_3 (Figure 1D). Although the concentration of 1,650 mg/L NH_4NO_3 resulted in multiple shoot induction, vegetative growth was comparatively low (Figure 1C). NH_4NO_3 deficient plants, including 0 mg/L NH_4NO_3 (Fig. 1A) and 825 mg/L NH_4NO_3 (Figure 1B) appeared stunted and pale light green or yellow due to restricted growth of the vegetative organs. The findings of this study agree with Barker and Pilbeam (2006) who reported that NH_4NO_3 deficiencies restrict the growth of plant organs, roots, stems, and leaves. Therefore, Jerusalem artichoke should be cultured in a suitable concentration of NH_4NO_3 to ensure good vegetative growth.

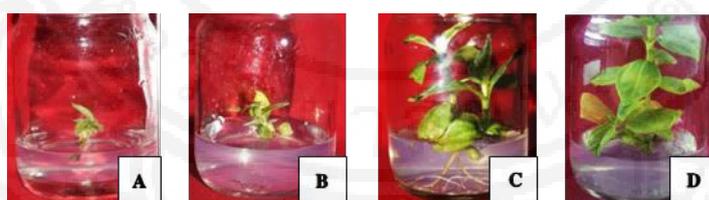


Figure 1 shows *in vitro* plantlet formation and growth characteristics of Jerusalem artichoke (*Helianthus tuberosus* L.).

Shoot regeneration from cotyledonary nodal segment growing on (A); MS without NH_4NO_3 (0 mg/L NH_4NO_3), (B); MS + $\frac{1}{2}$ NH_4NO_3 (825 mg/L NH_4NO_3), (C); MS + NH_4NO_3 (1,650 mg/L NH_4NO_3), (D); MS + 2 NH_4NO_3 (3,300 mg/L NH_4NO_3) after 4 weeks.

In plant tissue culture, NH_4NO_3 enhances plant growth due to the rapid synthesis of proteins and amino acids, which are further useful for carbohydrate synthesis. However, the rapid development of cells results in high hyperhydric causing abnormal plant stomata. Figure 2 shows the characteristics of hyperhydric shoots of Jerusalem



artichoke after 4 weeks of cultivation on MS medium containing sucrose (30 g/L), phytagel (12.4 g/L), and different NH_4NO_3 concentrations. Figure 2A shows formations of multiple shoots on plantlets in 1,650 mg/L NH_4NO_3 . While Fig. 2B shows deformed plantlets and large leaves due to rapid cell division on MS medium in 3,300 mg/L NH_4NO_3 . Therefore, as noted by George et al. (2008), a high concentration of 3,300 mg/L NH_4NO_3 on the culture medium increases the hyperhydricity.

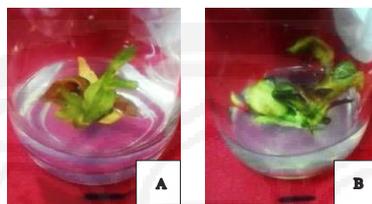


Figure 2 Hyperhydric shoots characteristics of Jerusalem artichoke (*Helianthus tuberosus* L.) *in vitro*. Hyperhydric shoots formation of Jerusalem artichoke after cultured on (A); MS + 1650 mg/L NH_4NO_3 , (B); MS + 3300 mg/L NH_4NO_3 for 4 weeks.

Conclusion

This study shows treatment of NH_4NO_3 in node culture of Jerusalem artichoke induces vegetative growth and inulin production. The results found a better vegetative growth of Jerusalem artichoke in 3,300 mg/L NH_4NO_3 (double concentration of NH_4NO_3 in MS medium), but it resulted in higher hyperhydricity due to the rapid development of cells causing abnormal plant. In addition, the study did not detect inulin in 3300 mg/L NH_4NO_3 because vegetative growth used NH_4NO_3 ; hence, there were no secondary metabolites. Leaving 1,650 mg/L NH_4NO_3 (normal concentration of NH_4NO_3 in MS medium) as the next best concentration of vegetative growth. Additionally, the experiment detected the highest inulin content of 3.07 g/100 g of dry weight in 1,650 mg/L NH_4NO_3 in the 3rd subculture. However, 3rd, 4th, and 5th subcultures in 0 mg/L NH_4NO_3 (no NH_4NO_3 concentration in MS medium) and 5th subcultures in 825 mg/L NH_4NO_3 (half concentration of NH_4NO_3 in MS medium) also detected some inulin production. Small amount of inulin content at this concentration level is to resist the stress caused by NH_4NO_3 deficiencies. Overall, 1,650 mg/L NH_4NO_3 was suitable for both vegetative growth and inulin production from Jerusalem artichoke because it is normal concentration NH_4NO_3 in MS medium that contain essential nutrients for plant growth, completely. Although, no NH_4NO_3 or half concentration in MS medium can induce inulin, the time required to subcultures is greater and the amount of inulin is less. The plantlets obtained from the culture are stunted. Therefore, they are not suitable for propagation.

Plant tissue culture using NH_4NO_3 improved the plant's physiological and secondary metabolites because NH_4NO_3 served as an important source of nitrogen for proteins, nucleic acids, and chlorophyll. George et al. (2008) also reported that NH_4NO_3 played an important role in plant's physiological changes, vegetative growth, and secondary metabolites. Therefore, this study recommended the utilization of NH_4NO_3 in plant tissue culture for better plant yield and quality of secondary metabolites. These findings will serve as a prototype for Jerusalem artichoke propagation and inulin production in the future.



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