

Effects of inorganic salts on biomass production, cell wall components, and bioethanol production in *Nicotiana tabacum*

Seon Jeong Sim · Seong Hyeon Yong · Hak Gon Kim · Myung Suk Choi · Pil Son Choi

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Abstract The development of bioenergy through biomass has gained importance due to the increasing rates of fossil fuel depletion. Biomass is important to increase the productivity of bioethanol, and production of biomass with high biomass productivity, low lignin content, and high cellulose content is also important in this regard. Inorganic salts are important in the cultivation of biomass crops for the production of biomass with desirable characteristics. In this study, the roles of various inorganic salts in biomass and bioethanol production were investigated using an in vitro tobacco culture system. The inorganic salts evaluated in this study showed dramatic effects on tobacco plant growth. For example, H_2PO_4 substantially improved plant growth and the root/shoot (R/S) ratio. The chemical compositions of tobacco plants grown in media after removal of various inorganic salts also showed significant differences; for example, lignin content was high after Mg^{2+} removal treatment and low after K^+ treatment and H_2PO_4 removal treatment. On the other hand, NO_3^- and H_2PO_4 treatments yielded the highest cellulose content, while enzymatic hydrolysis yielded the highest

glucose concentration ratio 24 h after NH_4^+ removal treatment. The ethanol productivity after H_2PO_4 removal treatment was 3.95% (w/v) 24 h after fermentation and 3.75% (w/v) after 36 h. These results can be used as the basis for producing high-quality biomass for future bioethanol production.

Keywords Enzymatic hydrolysis, Fermentation, Inorganic salts, in vitro culture, *Nicotiana tabacum* L.

Introduction

This is because lignocellulosic biomass is non-edible, can be continuously reproduced, and is environmentally friendly because it does not emit greenhouse gases during production and use (Chandel et al. 2018). It is estimated that about 130 gallons of ethanol can be produced from 1.3 ton of biomass (Carroll et al. 2009).

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin, making it difficult for enzyme hydrolysis. That is why some pretreatment steps are essential to alter the physical and/or chemical structure of lignocellulosic biomass and easily convert polysaccharides into fermentable sugars, making cellulose more accessible (Kumar and Sharma 2017).

To effectively use lignocellulosic biomass, structural factors representing cellulose specific surface area, cellulose crystallinity, degree of polymerization, pore size and volume, and chemical factors related to the composition and content of lignin, hemicellulose, and acetyl groups must be considered (Zoghalmi and Paës 2019).

Biomass is very important as a raw material for bioenergy, but the chemical composition of biomass is also important. Lignin imparts water resistance, firmness, and mechanical strength to the secondary cell wall, but interferes with the saccharification of cellulose. Lignin imparts water resistance, firmness and mechanical strength to the secondary cell

[†]These authors contributed equally to this paper (co-corresponding authors).

S. J. Sim · H. G. Kim
Forest Research Department, Gyeongsangnam-do Forest Environment Research Institute, Jinju, 52615, Korea

S. H. Yong · Myung Suk Choi[†]
Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Gyeongnam, 52828, South Korea

M. S. Choi[†] (✉)
Division of Environmental Forest Science, Gyeongsang National University, Jinju, 52828, Republic of Korea
e-mail: mschoi@gnu.ac.kr

P. S. Choi[†] (✉)
Department of Medicinal Plant Resources, Nambu University, Wealkye-dong, Gwangsan-gu, Gwangju 506706, Korea
e-mail: cps6546@hanmail.net

wall, but interferes with the saccharification of cellulose or xylan. Lignin is given a water-resistance, durability and mechanical strength of the second cell wall, but hinders the saccharification of cellulose or xylan. Therefore, a pretreatment process to remove lignin is required, and this process requires a lot of cost and time (Simmons et al. 2010).

The most ideal feedstock is one with a low lignin content and a high content of cellulose (Mandre (2002). However, these plant cell wall compounds are greatly affected by the cultivation environment. Soil nutrients must be carefully considered for high biomass production of bioenergy crops (Dimitriou and Rutz 2015). Plant growth is usually able to produce the most biomass in soil that contains a balanced amount of inorganic salts. Also, nutrients composition can cause a change in the content of lignin and other secondary metabolites in plants (Chapin 1991). The factors that can alter inorganic salts in plants may also affect the lignification process. However, the role of the inorganic salts in lignification is still not clear. The regulation of the supply of inorganic salts in plants may be one of the most easily practicable ways of directing the biosynthesis of lignin and other compounds in plants.

The lignocellulosic biomass is mainly composed of cellulose, hemicelluloses, and lignin, along with smaller amounts of pectin, protein and ash (Jørgensen et al. 2007). Pretreatment has been viewed as one of the most important and expensive steps in releasing sugars from hemicelluloses and cellulose (Kadam et al. 2008). Removing lignin and hemicelluloses, the reduction of cellulose crystallinity and the increase of porosity during the pretreatment processes can significantly improve the enzymatic hydrolysis of cellulose contained in the lignocellulosic material (Singh and Chen 2008).

The chemical and physical properties of the lignocellulosic biomass can affect the choice and efficiency of the biomass conversion process (Tillman 2000). However, very few studies have been made on the effects of inorganic salts on these lignocellulosic biomass. Especially in the field, these studies have been difficult to create a uniform environment. The purpose of this study is to evaluate the effect of inorganic salts on plant growth and biosynthesis of major compounds using the in vitro culture system of tobacco, a model plant, and further investigate the bioethanol production efficiency of each inorganic salts.

Materials and Methods

Plant materials and preparation of medium with revised

inorganic salts

The plant material for *N. tabacum* was provided by the National Institute of Forest Science. In vitro plants were passaged every 4 weeks in 1/2MS (Murashige and Skoog 1962) medium and cultured for 1 year. Plants used in the experiment were cultured for 6 weeks at a size of 5 cm in the early stage of culture. Cultured plants were harvested after 14, 28, 42 and 56 days of culture, respectively, and their growth characteristics were investigated. We used a plant with a size of 15 cm as the sample for chemical analysis.

The medium was prepared to investigate the effects of inorganic salts on biomass production, the major components of the biosynthesis of plants and cell walls and the bioethanol production of *N. tabacum*. In addition, in a previous study, it was found that 1/2 MS medium is suitable for culture, and the concentration used in this study was determined within this range.

The 1/2 MS solid medium was prepared by removing large amounts and trace inorganic salts. T1 is a basic medium composition for MS, and T2 to T8 are treatments in which nitrogen sources (ammonium and nitrate), potassium, calcium, magnesium, and iron are removed. Each treatment contains all of the other ingredients except inorganic salts. The revised medium was adjusted to pH 5.8 after the addition of 0.7% agar without the addition of growth regulators. The pH of the eight experimental treatments used in this study was 4.3–5.2. Therefore, it was neutralized with 1N HCl to reach an optimum pH of 5.8.

National Renewable Energy Laboratory (NREL Golden, CO) analytical methods for biomass (NREL 1996), carbohydrates, lignin (acid-insoluble, acid soluble), extractives and the ash content of raw materials were determined according to NREL procedures (Sluiter et al. 2008). Reagents used for all analysis were Sigma-Aldrich's extra pure grade.

In vitro culture in medium revised with inorganic salts

Surface-sterilized *N. tabacum* shoots were cut into 2–3 cm pieces and cultured in 1/2 MS solid medium. In vitro plantlets were further placed in culture vessels containing 50 mL of 1/2 MS basal medium containing sucrose (3% w/v). All cultures were maintained under a 16 h light/8 h dark photoperiod in a growth chamber fitted with a cool fluorescent light emitting 25 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR).

To determine variations in biomass by inorganic salt treatments, individual shoots were cultured on the revised

Table 1 Inorganic salt compositions of the media used for in vitro culture

Treatment	Medium inorganic salt (mM)						
	NH ₄ ⁺	NO ₃ ⁻	H ₂ PO ₄ ⁻	K ⁺	Ca ²⁺	Mg ²⁺	Fe ³⁺
T1	0.515	0.985	0.031	0.503	0.748	0.751	0.010
T2	0	0.470	0.031	0.503	0.748	0.751	0.010
T3	0	0	0.031	0.033	0.748	0.751	0.010
T4	0.515	0.985	0	0.472	0.748	0.751	0.010
T5	0.515	0.515	0	0	0.748	0.751	0.010
T6	0.515	0.985	0.031	0.503	0	0.751	0.010
T7	0.515	0.985	0.031	0.503	0.748	0	0.010
T8	0.515	0.985	0.031	0.503	0.748	0.751	0

1/2 MS solid medium without any plant growth regulators for 8 weeks. Shoot growth and biosynthesis of lignin, cellulose and low molecular weight compounds were investigated by culturing plants in 1/2 MS medium except for each inorganic salt. (Table 1).

After 8 weeks of culture, the number of shoots and leaves, wilting rate, fresh and dry biomass weight of above and underground, and root/shoot ratio were investigated. The chlorophyll content of plant leaves was measured using a Soil Plants Analysis Development (Soil Plants Analysis Development) 502 portable chlorophyll meter (Minolta Co., Ltd., Japan). The SPAD values were repeatedly taken at the center of the leaves throughout the experiments.

Lignin biosynthesis assay

For the histochemical analysis of lignin in biomass, the lignin of the cultured biomass was stained with Wiesner reagents (Geiger and Fuggerer 1979). Wiesner's reagent states that under acidic conditions, phloroglucinol produces a red-pink product with the cinnamaldehyde group present primarily in lignin. The shoots were sectioned with a sharp razor blade, and sections were placed in 2% phloroglucinol in 95% ethanol for 5 min and mounted in 6N HCl. The lignin was identified as a red color when observed under a light microscope.

Chemical composition of biomass obtained in different media

To determine the chemical composition of biomass, National Renewable Energy Laboratory analytical methods (NREL 1996) were applied. The shoot tissue (dry weight 100 g) was cut into pieces and vacuum-dried at 70°C for 24 hours. Thereafter, the sample was extracted with ethanol for 3 hours with a Soxhlet extractor and then vacuum dried at 105°C. for 48 hours. The lignin and holocellulose contents were measured after delignification with NaClO₂ (Wise et

al. 1946). 2.5 g of the degreased sample was repeatedly treated with 1 g of NaClO₂ in 0.2 mL of diluted acetic acid solution) three times, and then reacted at 70°C for 1 hour. The delignified product, holocellulose, was filtered, washed with distilled water, dried at 105°C for 48 h *in vacuo* and weighed.

The α -cellulose content was determined as the insoluble residue in the NaOH (17.5%) aqueous solution. A flask containing a 1 g sample of the holocellulose obtained according to the process above was dissolved in NaOH. The mixture was stirred for 30 min at 20°C, and 25 ml of distilled water was then added to the mixture. After 5 min, the residue was filtrated, and then supplemented with 40 ml of 10% acetic acid and allowed to stand. The resulting residue was collected by filtration and washed with 1 L of boiling water. The residue that contained α -cellulose was finally dried at 105°C for 48 h *in vacuo* and weighed. The contents of lignin, holocellulose, and α -cellulose in the original *N. tabacum* chips were calculated as dry weights relative to the original plant mass. The α -cellulose content was quantified as cellulose. The hemicellulose content of the biomass was determined by the subtraction of the α -cellulose from that of holocellulose. To observe cellulose using field emission scanning electron microscopy (Fe-SEM) analysis, delignificated tissues were air-dried before being used for microscopic examination. The α -cellulose solids were transferred to carbon tape on copper stubs and sputtered with gold (10 mA and 300s) using a high-resolution sputter coater (Agar Scientific Ltd, USA). Fe-SEM investigations were carried out with a Philips XL30 S-FEG Microscope (Netherlands) operated at 15 kV, Magnification 200x or 300x. Images were acquired with Quartz PCI Version 4 software (Hitachi high-technologies, Japan).

Enzymatic hydrolysis and ethanol fermentation

Enzymatic hydrolysis was carried out on the non-delignified

raw material. For enzymatic hydrolysis, 1 g of extractive-free dry biomass was finely ground and transferred to a 250 mL Erlenmeyer flask containing 50 mL of 0.1 M sodium citrate buffer (pH 4.8). Next, appropriate amounts of cellulase (58.56 FPUg⁻¹) and an equal amount of β -glucosidase (24.2 FBG/g) were added. The flask was placed in a shaking incubator (IS-97IR, Jeio-Tech Co. Korea) and maintained at 50°C for 36 h (150 rpm). The released glucose in the medium was analyzed with a mutarotase-GOD method employing a glucose CII-test kit (Wako Pure Chemical Industrie Ltd.) after the heat-denaturing of enzymes (100°C for 10 min). The enzymes used for enzymatic hydrolysis were Celluclast 1.5 L (Novo Co. Denmark), cellulase from *Trichoderma reesei* and Viscozyme L (Novo Co. Denmark) as β -glucosidase.

Regarding fermentation, *Saccharomyces cerevisiae* KCCM 11215 was used for the ethanol fermentation study. The inoculum required for fermentation was developed in YM (Yeast and Mold) liquid medium (yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L and dextrose 10 g/L). Later, an inoculum (1.5×10^8 cells/mL) was seeded to fermentation broth (5% v/v).

Ethanol production was quantified by a gas chromatograph (GC) (Agilent Technologies). The fermentation broth obtained after 36 h was filtered through a 0.2 μ m filter before GC analysis to remove any coarse particles (Krishna and Chowdary 2000) for alcohol estimation. The GC was equipped with an HP-FFAP column of 30 m with an ID 0.25 mm. The detector and injection port were maintained at 200°C. The combustion gas was a mixture of hydrogen and air, and nitrogen was used as a carrier gas at a flow

rate of 40 mL/min. The quantification of ethanol by the GC was obtained by comparison with a standard curve generated based on the detector response to known amounts of alcohol.

Statistical analysis

A one-way ANOVA was used to test for differences in *N. tabacum* growth. Means were separated by Duncan's multiple range test ($P = 0.05$). Mean values were expressed as standard error (\pm SE). All data sets were subjected to analysis with the SPSS package (SPSS, Inc. Chicago, IL, USA).

Results

Effect of inorganic salts on shoot growth

Inorganic salt had a dramatic effect on tobacco plant growth (Table 2). The control without changing the inorganic salt component had the best growth. After 8 weeks of culture, the sprout growth of tobacco in the control was 12.45 cm. The treatments that showed the best growth in the inorganic salt removal medium was the Ca⁺ treatment, followed by Fe³⁺ and Mg²⁺. On the other hand, the treatment that most inhibited the growth of tobacco shoots was H₂PO₄, and almost no growth was achieved. In addition, treatments that inhibited growth were in the order of NH₄⁺, K⁺, and NO₃⁻.

Table 2 Influence of various inorganic salt treatments on plants

Treatment	Shoot growth		Withering rate		Leaf appearance		SPAD chlorophyll value	Root growthb (cm)
	Shoot growth	No. of shoots/explants ^b	No. of leaves	Withering rate	Leaf length	Leaf width (cm)		
T1	12.64±0.23a	1.00±0.00	19.8±1.08a	1±0.61b	3.31±0.52d	2.34±0.34b	31.83±5.08a	6.91±0.93bc
T2	5.74±1.08d	1.20±0.22	12.8±0.89d	1.4±0.57b	3.88±0.14cd	2.33±0.09b	23.92±5.93b	9.13±2.76ab
T3	8.11±1.65c	1.40±0.45	16.75±1.31c	0.75±0.48b	4.33±0.5bc	2.63±0.23b	15.8±0.99c	3.46±0.26d
T4	1.88±0.21e	1.00±0.00	7±1e	4.6±0.76a	2.02±0.08e	1.32±0.14c	2.23±0.98d	9.3±1.25a
T5	7.03±0.98c	1.40±0.27	18.2±0.96bc	6.6±1.3a	3.62±0.12d	2.44±0.06b	17.9±3.75bc	6.64±0.48c
T6	10.75±0.56b	1.00±0.00	17.2±2.41c	5.2±2.54a	5.66±0.31a	3.44±0.31a	18.03±4.41bc	7.46±0.33abc
T7	10.6±0.58b	1.00±0.00	16±1.47c	4.75±1.89a	4.51±0.54bc	3.11±0.25a	20.05±3.67bc	6.89±1.33bc
T8	10.52±1.31b	1.00±0.00	17.33±1.26bc	1.33±0.58b	4.78±0.36b	3.37±0.24a	31.73±2.39a	7.21±0.7abc

*T1 (Control, BFM; Basal Formulation Medium); T2 (BFM without NH₄⁺); T3 (BFM without NO₃⁻); T4 (BFM without H₂PO₄⁻); T5 (BFM without K⁺); T6 (BFM without Ca²⁺); T7 (BFM without Mg²⁺); T8 (BFM without Fe³⁺). **Each value represents the mean±standard error of at least three replicates.

Appearance of plant and lateral shoot induction

Inorganic salts influenced the formation of multiple shoots of *N. tabacum* (Table 2). Multiple shoot was highly induced in NO_3^- and K^+ removal treatments. The 1/2MS medium from which the NO_3^- component was removed produced a significantly higher number of shoots than the other media tested. However, other than the control, nitrogen source and K^+ removal treatment, multiple shoots were not induced.

Effect of inorganic salts on leaf appearance

Inorganic salt affects the development and appearance of leaves (Table 2). Plant leaves in the inorganic salt removed medium had fewer leaves than in the control medium (1/2 MS basal medium) without inorganic salt adjustment. The number of leaves was the highest in the medium without K^+ , but the number of leaves was the least in the in the medium without H_2PO_4^- . However, plant leaves cultured in Ca^{2+} and Fe^{3+} removal media appeared longer, although transient. Leaf length had no significant effect except for H_2PO_4^- removal media. However, plant leaves cultured in Ca^{2+} and Fe^{3+} removal media appeared longer, although transient.

Leaf width also affected the inorganic salt removal medium. Leaf width also showed similar pattern to leaf length. Leaf width was very small in Ca^{2+} removal medium but had no significant effect in other treatments. Rather, the leaf width was larger in the Ca^{2+} , Mg^{2+} and Fe^{3+} removal media.

Leaves cultivated in the inorganic salt removed medium were observed to wilt over time. In particular, in the

treatment of removing K^+ and Ca^{2+} , the wilt rate of plants was high, but in the treatment of removing NO_3^- , leaf wilt was less. At the tips and edges of the leaves, mainly after yellowing of the leaves, necrosis was observed (Table 2).

The chlorophyll content of the leaves also had a large amount depending on the type of inorganic salt. The measured SPAD value was the lowest in the NO_3^- removal treatment, followed by H_2PO_4^- and K^+ . However, the SPAD value of leaves was higher than that of the control treatment in the Ca^{2+} removal treatment, indicating that Ca^{2+} did not significantly affect the chlorophyll content. In addition, it was found that the SPAD value of the leaf was not significantly affected by Mg^{2+} and Fe^{3+} ions.

Effect of inorganic salts on root growth and S/R ratio

Root growth was different according to the type of inorganic salt (Table 2). In the control, root formation occurred within 56 days, and 100% roots occurred in the inorganic salt removal medium (data not shown). Root induction and growth also affected according to inorganic salt kinds. It was found that K^+ and Mg^{2+} had a great influence on root development and growth.

Effect of inorganic salts on the aboveground and underground biomass production

Changes in biomass production of plants cultured for 8 weeks in a medium from which inorganic salts were removed were investigated (Table 3). Inorganic salts have affected the biomass production of tobacco cultured in vitro. Overall, except for the Fe^{3+} -removed treatments, the

Table 3 Effect of inorganic salts on biomass production in *N. tabacum*

Treatment ^a	Fresh mass production (g/plant)			Dry mass production (g/plant)			R/S ratio ^b
	Aboveground part	Underground part	Total	Aboveground part	Underground part	Total	
T1	5.40	0.47	5.87	0.29	0.04	0.33	0.14
T2	2.45	0.59	3.04	0.11	0.04	0.15	0.36
T3	2.79	0.35	3.14	0.27	0.02	0.29	0.08
T4	0.45	1.37	1.82	0.07	0.09	0.16	1.29
T5	2.55	1.01	3.56	0.38	0.08	0.46	0.21
T6	4.87	1.50	6.37	0.41	0.13	0.54	0.32
T7	4.55	1.83	6.38	0.41	0.13	0.54	0.32
T8	5.89	1.85	7.74	0.41	0.15	0.56	0.36

^aT1 (Control, BFM); T2 (BFM without NH_4^+); T3 (BFM without NO_3^-); T4 (BFM without H_2PO_4^-); T5 (BFM without K^+); T6 (BFM without Ca^{2+}); T7 (BFM without Mg^{2+}); T8 (BFM without Fe^{3+}).

^bR/S ratio = Root dry weight (g)/Shoot dry weight (g).

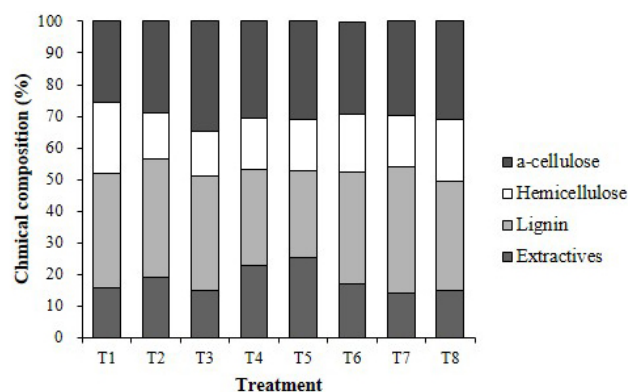


Fig. 1 Chemical composition (%) based on inorganic salt treatment of *in vitro* cultivated biomass. T1 (Control, BFM); T2 (BFM without NH_4^+); T3 (BFM without NO_3^-); T4 (BFM without H_2PO_4^-); T5 (BFM without K^+); T6 (BFM without Ca^{2+}); T7 (BFM without Mg^{2+}); and T8 (BFM without Fe^{3+})

aboveground biomass productivity of tobacco decreased. The treatment with the lowest biomass production on the aboveground was the H_2PO_4^- removal treatment, and the production was very low in the NH_4^+ and NO_3^- removal treatment. The biomass production in the underground was different from that of the above-ground (Table 3). The production of biomass underground increased in other treatments except for the medium from which NO_3^- was removed. In particular, the Mg^{2+} and Fe^{3+} removal treatments produced 3.9 times that of the control treatment. The standard biomass production during drying also showed a similar trend to that of fresh.

In addition, the ratio of the amount of biomass in the aboveground and the amount of biomass in the basement was different in the inorganic salt treatment (Table 3). The R/S ratio of the control treatment was 0.13, but the R/S ratio was increased in all except for the NO_3^- removal treatment. The treatment with the highest R/S ratio was the treatment with H_2PO_4^- removal.

Effect of inorganic salts on the chemical composition

The chemical composition of tobacco plants grown in inorganic salt-free medium showed significant differences between treatments (Fig. 1). Extractive content varied from treatment to treatment. Treatment with the highest extractive content was K^+ , followed by H_2PO_4^- , NH_4^+ , and Ca^{2+} .

The lignin content also showed differences according to the inorganic salt (Fig. 1). The treatment with the lowest lignin content was the K^+ treatment, followed by the H_2PO_4^- removal treatment.

Histochemical observation of lignin according to the inorganic salt treatment was performed (Fig. 2). Because

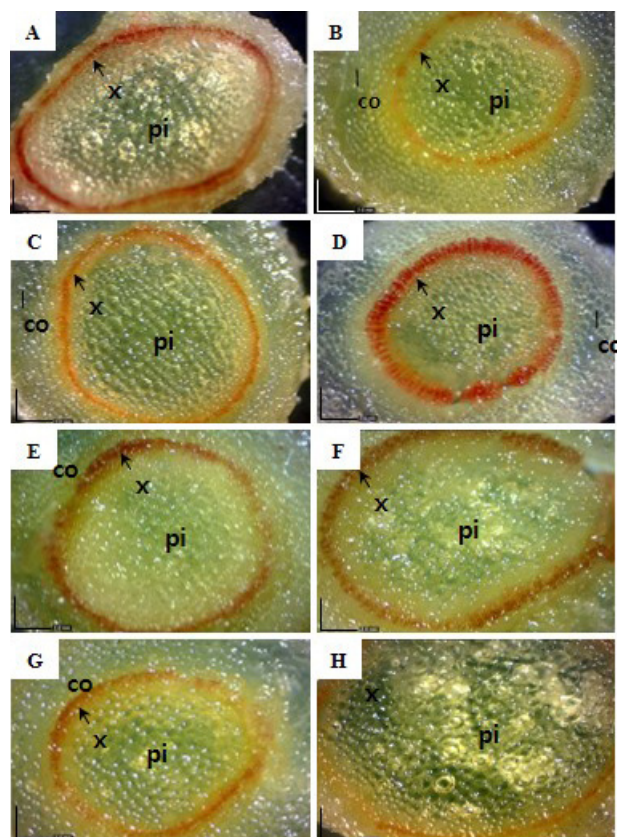


Fig. 2 Histochemical lignin (phloroglucinol-HCl) staining of shoot cross-sections from 8-week-old *N. tabacum* plantlets with Wiener (bar: 2 mm). Lignin is stained red. Abbreviations: co, cortex; pi, pith; x, xylem. Magnification, x300. (A) T1 (Control, BFM); (B) T2 (BFM without NH_4^+); (C) T3 (BFM without NO_3^-); (D) T4 (BFM without H_2PO_4^-); (E) T5 (BFM without K^+); (F) T6 (BFM without Ca^{2+}); (G) T7 (BFM without Mg^{2+}); and (H) T8 (BFM without Fe^{3+}).

Wiesner reagent gives only temporary visualization of color, images were recorded within 10 min of reagent application. To visualize the lignified tissues in 8 weeks-old plantlet shoots, cross section of middle was treated with the Wiesner (phloroglucinol-HCl) reagents. The presence of lignin was confirmed by phloroglucinol-HCl staining. The amount of lignin dyeing was different for each inorganic salt removal treatment. The staining area of lignin begins in the phloem fibers (pf) and in the neck (x). Among them, the most distinct treatments were the H_2PO_4^- removal treatments. However, the degree of occurrence between treatments was not significantly different.

Hemicellulose content also varied from treatment to treatment (Fig. 1). The hemicellulose content of the plants in the inorganic salt removal medium was 22.40%, which was lower than that of the plants grown in the control medium. The treatment with the lowest hemicellulose content was 14.52% with NO_3^- , and the treatment with NH_4^+ and

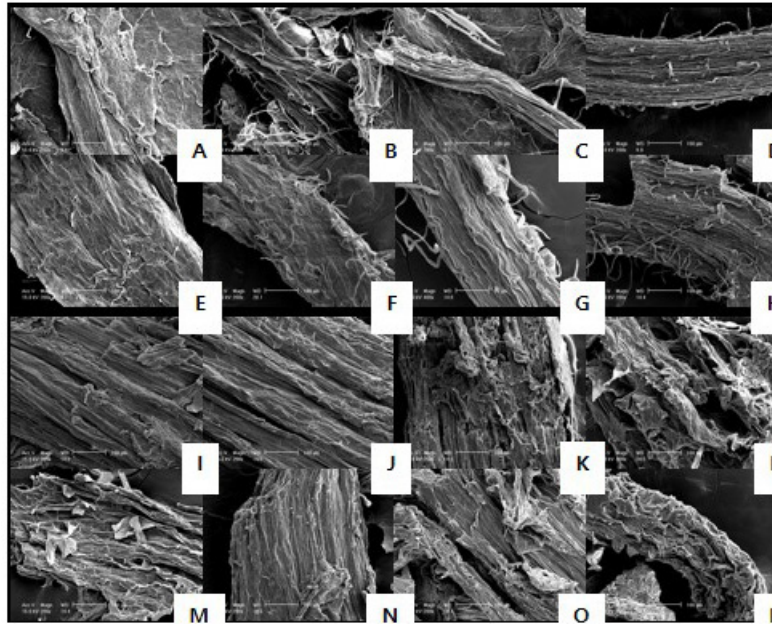


Fig. 3 Fe-SEM images after inorganic salt treatments of the dried alphacellulose of *N. tabacum* (I-P). (A and I) T1 (Control, BFM); (B and J) T2 (BFM without NH_4^+); (C and K) T3 (BFM without NO_3^-); (D and L) T4 (BFM without H_2PO_4^-); (E and M) T5 (BFM without K^+); (F and N) T6 (BFM without Ca^{2+}); (G and O) T7 (BFM without Mg^{2+}); and (H and P) T8 (BFM without Fe^{3+}).

H_2PO_4^- removal also had a low content. Among the inorganic salt removal treatments, the treatment with a low hemi-cellulose content was Fe^{3+} .

The α -cellulose content was also different according to the inorganic salt treatments (Fig. 1). The α -cellulose content of plant varied from 25.71% to 34.63%. It was found that the cellulose content of plants cultured in the treatment from which the inorganic salt was removed was increased compared to the control. Among them, the treatment with the highest cellulose content was NO_3^- treatment, followed by H_2PO_4^- and K^+ treatment. To investigate the morphology of cellulose purified from plants cultured in an inorganic salt removal medium, it was analyzed by Fe-SEM (Fig. 3). Microscopic analysis helped to visualize clear surface morphological differences between α -cellulose samples. It was found that cellulose microfibrils were exposed on the surface of the biomass. The SEM image suggested that the main component present in the original microcrystalline cellulose was coarse and condensed fibrils. Cellulose cultured in the inorganic salt removal medium was coarser. However, there was no significant difference in the structure of the cellulose of plants grown in each treatment group only by SEM analysis.

Effect of inorganic salts on enzymatic hydrolysis

As a result of enzymatic hydrolysis of plants grown in inorganic salt removal medium, it was found that the

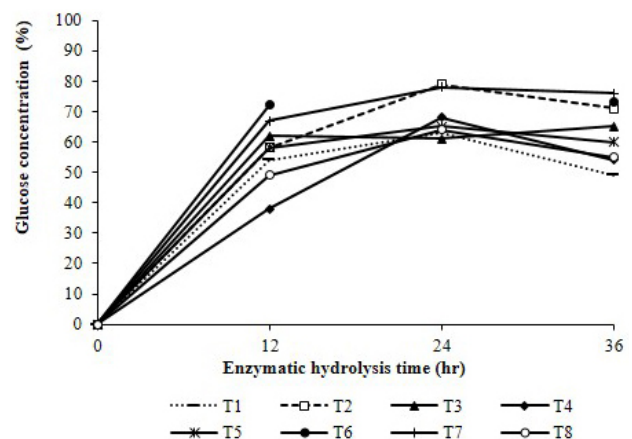


Fig. 4 Effect of time on enzymatic hydrolysis of *N. tabacum* after inorganic salt treatment Temperature, 50°C; pH 4.8; enzyme loading, 58.56 FPU·g⁻¹ of cellulose (Celluclast 1.5 L) and 24.2 FBG/g of β -glucosidase (Novozyme). T1 (Control, BFM); T2 (BFM without NH_4^+); T3 (BFM without NO_3^-); T4 (BFM without H_2PO_4^-); T5 (BFM without K^+); T6 (BFM without Ca^{2+}); T7 (BFM without Mg^{2+}); and T8 (BFM without Fe^{3+}).

saccharification rate increased with time (Fig. 4). In addition, the saccharification rate showed a large difference according to the inorganic salt removal treatments. The treatment with the highest saccharification rate reached the highest concentration ratio of glucose after 24 hours of enzymatic hydrolysis for NH_4^+ removal treatment and Mg^{2+} removal tobacco treatment, respectively, 79% and 78%.

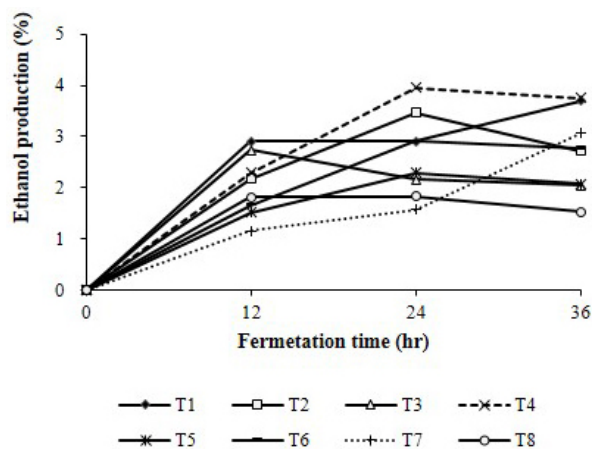


Fig. 5 Effect of time on ethanol production after treatment of *N. tabacum* with inorganic salts. Enzymatic saccharification was performed using 58.56 FPU of cellulose (Celluclast 1.5 L) and the cellulose proportion of β -glucosidase (Novozyme 188). T1 (Control, BFM); T2 (BFM without NH_4^+); T3 (BFM without NO_3^-); T4 (BFM without H_2PO_4^-); T5 (BFM without K^+); T6 (BFM without Ca^{2+}); T7 (BFM without Mg^{2+}); T8 (BFM without Fe^{3+}).

Effect of inorganic salts on fermentation of enzymatic hydrolyzates

The fermentation process was carried out using glucose obtained by enzymatic hydrolysis. *S. cerevisiae* KCCM 1215 was also used. Ethanol productivity was different for each treatment (Fig. 5). After the 36th period of fermentation, the highest ethanol production was found in the H_2PO_4^- removal treatment, and NH_4^+ and K^+ removal treatment. Ethanol productivity in the H_2PO_4^- removal treatment was 3.95% (w/v) after 24 hours of fermentation and 3.75% (w/v) at 36 hours. On the other hand, the treatment with the lowest ethanol production was found to be Fe^{3+} removal treatment. Ethanol production was found to increase up to 24 hours of fermentation but not significantly after that.

Discussion

The effect of inorganic salts on biomass and bioethanol production from tobacco as herbaceous model plants was determined. Tobacco shoot had almost no effect on growth when cultured in a medium in which Ca^+ , Fe^{3+} and Mg^{2+} components were removed, but H_2PO_4^- and NH_4^+ removal treatment was good for growth. These results indicate that H_2PO_4^- and nitrogen sources (NH_4^+ and NO_3^-) are the major inorganic salts important for the growth of tobacco. Phosphoric acid is well known as the main inorganic salt

that increases cultivation and growth. Phosphorus is essential and is involved in the transfer of energy through ATP. It is also involved in root development and metabolic activity, especially in protein synthesis (Tanwar and Shaktawat 2003). In addition, the nitrogen NO_3^- -N and NH_4^+ N forms in the soil are absorbed by plants and used for plant growth. Deficiency of nitrogen can impair plant growth and root growth (Mengel and Kirkby 1987). However, it can be seen that components such as Ca^+ , Fe^{3+} and Mg^{2+} are relatively less involved in growth than N, P, and K.

The increase of a specific inorganic salt concentration can stimulate cell and tissue growth, but it can at the same time induce deficiencies of other nutrients by the dilution effect (Marschner 1995). Varying strength of inorganic salts of the basal media have been shown to markedly influence the micropropagation process in many plant species and suitable basal medium composition should be investigated for multiple shoots induction (Khalafalla et al. 2007).

Appearance of leaf was different based on each treatment. Leaf length had no significant effect except for the H_2PO_4^- removal medium. Leaf width also showed similar pattern to leaf length. These results mean that inorganic salts do not significantly affect leaf size. In general, it is known that the deficiency of inorganic salts reduces the size of leaves, which leads to a decrease in photosynthesis and eventually inhibits growth (Yeh et al. 2000). However, the results of this study were different from those of previous studies. The reason seems to be the short study period. Chlorophyll level in two species was affected based on inorganic salt treatments. The Ca^{2+} removal treatment had the highest average SPAD value. This result indicated that Ca^{2+} was not effect of inorganic salt on tobacco. The H_2PO_4^- and NO_3^- removed treatment had the lowest average SPAD value. As these plants carry out lower levels of photosynthesis, they eventually accumulate less cellular material and less carbon skeleton (Borgatto et al. 2002). Root induction and growth also affected according to inorganic salts. It was found that K^+ and Mg^{2+} had a great influence on root development and growth. Niu et al. (2014) reported that primary root elongation and lateral root formation in *Arabidopsis* were not influenced by low Mg^{2+} but inhibited by high Mg^{2+} after one-week period. Damm et al. (2011) suggested that low Mg^{2+} availability together with high K^+ supply enhanced the root-shoot ratio prior to any visible sign of Mg^{2+} deficiency symptoms in rice leaves.

The R/S ratio was also affected by the inorganic salt treatment. Under normal conditions, the ratio of root shoots in most trees ranges from 1:5 to 1:6 (Perry 1982). An increase

in soil fertility is generally associated with a decrease in the R/S ratio. That is, sprout growth gains more weight than root growth (Coutts and Philipson 1980). In the case of this study, the R/S ratio was the highest at about 1.00 in the K^+ removed medium. K^+ is an essential nutrient for plant growth and development. It is the most abundant cations in plant cells and can make up up to 10% of the dry weight of the plant (Véry and Sentenac 2003).

The dry matter of shoot and roots of tobacco under inorganic salt removal medium differ significantly from control treatment. Yield reduction may be associated not only to the toxicity of a given inorganic salts, but also to the deficiency of other inorganic salts causing an ionic unbalance among nutrients (Marschner 1995).

The content of compounds in the plant was dependent on the inorganic salt. Low molecular weight extracts differed from treatments, and treatments with the highest extract content were in the order of K^+ and $H_2PO_4^-$. The extract contents may contain non-structural components of biomass such as waxes, fats, tannins, sugars, some resins and coloring substances (Jung et al. 2010).

Inorganic salt treatment has been shown to play an important role in lignin biosynthesis. In particular, it is known that the lignin content is increased in plants cultured in K^+ removal medium. Mandre (2002) reported a strong correlation between lignin and Ca and K concentrations in *Picea* needles and shoots. In addition, an increase in K^+ concentration in wheat plants stimulated lignification (Miidla 1989). Hojatti and Maleki (1972) reported that K^+ increased the methionine content of wheat and that L-methionine could be a precursor to the methylation process during lignin formation.

Phloroglucinol staining has long been utilized for localizing lignins in plant tissue sections (Dean 1997). It stains lignins a purple coloration deriving from reactions of hydroxycinnamyl aldehyde end-groups that are systematically present in native lignin, albeit in low amounts (Jehan et al. 1994). Because Wiesner reagent gives only temporary visualization of color, images were recorded within 10 min of reagent application. Red staining with phloroglucinol indicates the presence of hydroxycinnamaldehydes in lignin. Phloroglucinol stains lignified cells red upon reaction with hydroxycinnamaldehyde groups present in the polymer. Herbal products such as tobacco have already reported lignin confirmation. Franke (2000) confirmed lignin in poplar and tobacco transformed with the ferulate 5-hydroxylase gene involved in lignin biosynthesis by the Wiesner method, which is consistent with this study.

Inorganic salt treatment was found to have no significant

effect on lignin content, but high concentration of inorganic salt treatment inhibited lignin biosynthesis in the poplar (Pitre et al. 2007).

Inorganic salts influenced the chemical composition of the tobacco plant cultured *in vitro*. The most ideal chemical composition for bioethanol production is lignin content is low, and the cellulose content is high. In this study, the lignin content was low in the plants cultured in the medium from which K^+ and $H_2PO_4^-$ were removed.

On the other hand, the cellulose content was highest in the NO_3^- and $H_2PO_4^-$ removing treatments. The α -cellulose level was found to be higher in $H_2PO_4^-$ removed treatments than control.

The inorganic salt treatment also affected the cellulose content. In this study, the cellulose content of plants cultured in a medium excluding a nitrogen source was increased. A number of studies have reported that inorganic salts affect cellulose biosynthesis. Nitrogen in trees or soil is considered an important factor for both lignin and cellulose biosynthesis. For example, high N fertility increases the cellulose concentration in *Pinus palustris* Mill. seedlings, resulting in lower lignin concentrations (Tullus et al. 2009).

Ultrastructure of cellulose also affected on inorganic salts kinds. Fe-SEM graphs indicate that the microfibrils are separated from the initial connected structure and fully exposed. It would certainly increase the external surface area and the porosity of the biomass. More accessible external and internal surface area of α -cellulose was attainable as binding site for cellulose, leading to the enhancement of enzymatic saccharification (Singh et al. 2009).

Inorganic salts were affected on enzymatic hydrolysis on *in vitro* cultured tobacco. In the treatment with the highest glycation rate, the concentration ratios of 79% and 78% of glucose were the highest after 24 hours of enzymatic hydrolysis for NH_4^+ removal treatment and Mg^{2+} removal tobacco treatment, respectively. Lignocellulosic biomass cannot be saccharified by enzymes to high yields without a pretreatment mainly because the lignin in plant cell walls forms a barrier against enzyme attack (Sewalt et al. 1997). The inorganic salt treatments would reduce the lignin content and crystallinity of the cellulose and increase the surface area. Factors that have been found to influence the hydrolysis of cellulose include the porosity of the waste (accessible surface area), cellulose fiber crystallinity, and lignin and hemicellulose content (McMillan 1994). As Marschner (1995) points out, potassium-deficient plants accumulate carbohydrates and soluble nitrogen compounds.

Yeast fermentation of raw sugars from tobacco plants

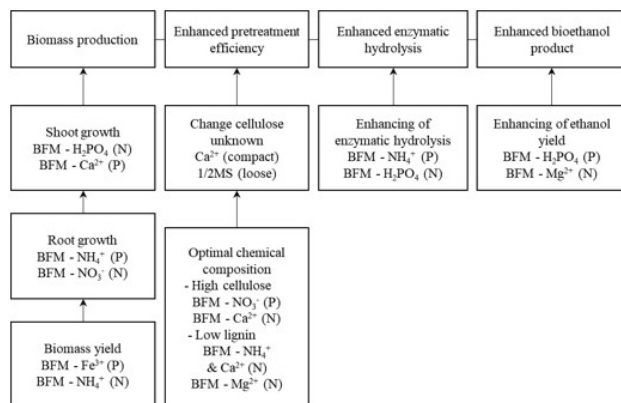


Fig. 6 Processing pathway for bioethanol production with cultured *N. tabacum*. Ethanol production involves biomass pretreatment, hydrolysis for monosaccharide production, and fermentation to produce ethanol. BPM; Bioenergy production medium, P: Positive; N : Negative

cultured in inorganic desalination medium has been shown to be effective. After 36 hours of fermentation, ethanol production was the highest in H₂PO₄⁻ removal treatment and NH₄⁺ and K⁺ removal treatment. In particular, in this study, although the sample used for saccharification was not pretreated, the ethanol yield was 3.95% (w/v) after 24 hours fermentation in H₂PO₄⁻ removal medium. After that, it is considered that bioethanol can be produced more economically if it is cultivated in a well-managed field of nutrition such as appropriate inorganic salts and proper pretreatment.

The ethanol productivity and fermentation time of this study were different from other previous studies. Studies on the production of bioethanol from most biomass (Spangler and Emert 1986) were fermented for 4–6 days and up to 2. It is said that the yield of 3.95% (w/v) ethanol was shown. The difference from our study is that the material used in this study was less wood formation.

In conclusion, proper selection of inorganic salts can control biomass production, changes in chemical composition, enzymatic hydrolysis, and ethanol production. Currently, processes for the production of biomass and bioethanol include pretreatment, saccharification and fermentation (Fig. 6). The use of appropriate inorganic salts can affect the loosening of the structural integrity of the biomass components (lignin-hemicellulose-cellulose), accelerate hydrolysis and reduce the cost of producing precursors for bioethanol production. Understanding the relationship between nutritional requirements and growth stages allows growers to tailor their fertilization program based on the type of soil. These results can be used as basic data for the development of appropriate techniques for the control

of various fertilizers for high biomass and bioethanol production in the future.

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