Influence of inorganic salts on biomass production, biochemical composition, and bioethanol production of *Populus alba*

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Inorganic salts are very important for the biosynthesis of major components such as cellulose and lignin. In order to investigate biomass production, major components of the biosynthesis of plant cell wall and the bioethanol production of *Populus alba*, we examined the effect of inorganic salts on *in vitro* culture systems without specific mineral salts. The medium without H₃PO₄ was supportive for *Populus alba* shoot growth, while the absence of NH₄⁺ resulted in poor shoot growth. The medium without H₃PO₄ and Fe⁺⁺ inhibited aboveground biomass production, whereas NH₄⁺ and K⁺ deprivation led to an enhancement of the same. The root/shoot ratio of *Populus alba* in the medium without H₃PO₄ was high compared with plants cultured in the control medium. H₃PO₄ is deeply involved in lignin biosynthesis, and its removal has been shown to reduce the biosynthesis of lignin. Plants grown on nitrate-free medium were found to be good for enzymatic saccharification and ethanol production. The plants grown in the medium without NO₃⁻ showed 72.0% enzyme digestibility, and the yield of ethanol showed 9.58% ethanol productivity after 12 hours. 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, *Populus alba*
loses 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 & Chen 2008). Many studies concerning the pretreatment step have been performed, such as the use of liquid hot water, dilute sulfuric acid, alkaline pretreatment ammonia fiber explosion (AFEX) and ammonia recycling (ARP – Hu & Ragauskas 2016). However, these pretreatment methods are still inefficient, highly expensive and not environmentally-friendly. Recently, ionic liquids (ILs) have been proven to be effective as solvents for lignocellulose pretreatment. This method results in the rapid saccharification of cellulose and hemicellulose, but it is too expensive, which remains a major obstacle to its commercialization. Therefore, it is very important that the content of lignin in the plant is low and the content of cellulose is high.

Variations in the chemical composition and physical properties of wood depend on plant species and the growth conditions related to their location (Mandre 2002), light environment (Peac & Sims 1994), temperature (Waring 1991) and the availability of nutrients for trees (Field & Mooney 1986).

The chemical and physical properties of the feedstock can affect the choice and efficiency of the biomass conversion process (Tillman 2000). The purpose of this study was to evaluate the effects of inorganic salts on the growth of poplar and biosynthesis of major compounds in plants using an in vitro culture system and to investigate the bio-ethanol production efficiency of poplar grown from each inorganic salt.

## Materials and methods

### Plant materials and preparation of medium with revised inorganic salts

The plant material of *P. alba* was distributed from the National Institute of Forest Science and planted at the Forestry Education Center of Gyeongsang National University (South Korea). Two-year-old seedlings were sampled and used for in vitro cultures. In vitro plants were subcultured every 4 weeks in ½ MS medium (Murashige & Skoog 1962) and cultured for 1 year. Plants used in the experiment were cultured for 6 weeks at a height of 5 cm in the early stage of culture. We used a plant with height 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 *P. alba*. An MS medium was used for the in vitro culture of white poplar (Lambardi et al. 2000). In addition, in a previous study, it was found that ⅓ MS medium is suitable for culture, and the concentration used in this study was determined within this range.

The ½ MS solid medium was prepared by removing large amounts and trace inorganic salts. Ti 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 (Tab. 1). 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 NH₄Cl to reach an optimum pH of 5.8.

### Lignin biosynthesis assay

For the histochemical analysis of lignin in biomass, the lignin of the cultured biomass was stained with Wiesner reagents (Geiger & Fuggerera 1979). The poplar shoots were sectioned freehand with a sharp razor blade, and sections were stained with Wiesner reagents. The lignin component was identified as a red color when observed under a light microscope.

### Chemical composition of *P. alba* biomass obtained in different media

To determine the chemical composition of biomass, National Renewable Energy Laboratory analytical methods (NREL 1996) were applied. The shoot tissues (100 g dry weight) were chopped into small pieces and dried at 70 °C for 24 h in vacuo, extracted with ethanol in a Soxhlet extractor for 3 h and dried at 105 °C for 48 h in vacuo. Lignin and hemicellulose contents were determined after delignification with NaClO₂ (Wis et al. 1946); briefly, a 2.5 g biomass of defatted *P. alba* was repeatedly (three times) treated with 1 g of NaClO₂, in dilute acetic acid solution (0.2 mL) at 70 °C for 1 h.

The defilgrified product, hemicellulose, was filtered, washed with distilled water, dried at 105 °C for 48 h in vacuo and weighed. The a-cellulose content was determined as the insoluble residue in the NaOH (17.5%) in vitro culture in medium revised with inorganic salts.

The *P. alba* stems were surface sterilized, segmented into 2–3 cm pieces and placed on ½ MS solid medium to allow growth. In vitro plantlets were further placed in culture vessels containing 50 mL of ½ 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 μmol m⁻² s⁻¹ of photosynthetically active radiation (PAR).

To determine variations in biomass by inorganic salt treatments, individual shoots were cultured on the revised ⅓ MS solid medium without any plant growth regulators for 8 weeks. The effect of inorganic salts on the shoot growth and biosynthesis of major compounds on the plantlets was studied by culturing *P. alba* on ½ MS medium free from one inorganic salt at a time (Tab. 1).

The shoot growth and growth appearance were recorded after 8 weeks of culture. The number of shoots and leaves, withering rate, and fresh and dry biomass weights were divided into above and below-ground, and the root/shoot rates were calculated according to the measured values.

The leaf chlorophyll content in nutrient-treated plants was measured using an SPAD (Soil Plants Analysis Development) portable chlorophyll meter (Minolta Co., Ltd., Japan). The SPAD values were repeatedly taken at the center of the leaves throughout the experiments.

### Tab. 1. Mineral salt compositions of media for in vitro culture of *P. alba* used in this study.

| Medium | NH₄⁺ | NO₃⁻ | H₂PO₄⁻ | K⁺ | Ca²⁺ | Mg²⁺ | Fe³⁺ |
|--------|------|------|--------|----|------|------|------|
| T1     | 0.515| 0.985| 0.031  | 0.503| 0.748| 0.751| 0.010|
| T2     | 0    | 0.985| 0.031  | 0.503| 0.748| 0.751| 0.010|
| T3     | 0.515| 0    | 0.031  | 0.503| 0.748| 0.751| 0.010|
| T4     | 0.515| 0.985| 0      | 0.503| 0.748| 0.751| 0.010|
| T5     | 0.515| 0.985| 0.031  | 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    |
aqueous solution. A flask containing a 1 g sample of the holocellulose obtained ac-
cording to the process above was dis-
solved in NaOH. The mixture was stirred for 30 min at 20 °C, and 25 mL of distilled 
water was then added to the mixture. Af-
ter 5 min, the residue was filtrated, and 
then supplemented with 40 mL of 10% 
acetic acid and allowed to stand. The re-
sulting residue was collected by filtration 
and washed with 1 L of boiling water. The residue that contained α-cellulose was fi-
nally dried at 105 °C for 48 h in vacuo and 
weighed. The content of lignin, holocellu-
lose, and α-cellulose in the original P. alba 
chips were calculated as dry weights rela-
tive to the original plant mass. The α-cellu-
lose content was quantified as cellulose. The hemi-cellulose 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, delignified tissues were air-
dried before being used for microscopic 
examination. The α-cellulose solids were 
transferred to carbon tape on copper stubs 
(Agar Scientific Ltd., Stansted, UK). Fe-SEM 
investigations were carried out with a Philips 
XL30 S-FeC® Microscope (Nether-
lands) operated at 15 kV, Magnification 
200× or 300×. Images were acquired with 
Quartz PCI® Version 4 software (Hitachi 
high-technologies, Japan).

Enzymatic hydrolysis and alcohol 
fermentation

Enzymatic hydrolysis was carried out on 
the non-delignified raw material. For enzy-
matic 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 
solution were added to the medium. The 
non-delignified raw material. For enzy-
meric hydrolysis and alcohol 
fermentation

Results and discussion

The appearance of shoot growth in 
medium revised with inorganic salts
The growth of P. alba was influenced by the presence of different inorganic salts in the 
medium to varying extents (Tab. 2). Major variations were observed with re-
spect to leaf color, leaf width, shoot num-
brer and plant height. The leaf color 
changed to yellow in the absence of K+ in 
the medium. Poplar stems were fairly well 
proliferating when cultured in media free 
from Ca2+. The leaves of poplar were ob-
served to be wider in NH4+ and Mg2+ defi-
cient mediums; however, the removal of 
NO3- and H2PO4 from growth medium pro-
duced narrow plant leaves with the pas-
sage of time. The pattern of shoot growth in the 
presence or absence of various inorganic salts 
is shown in Tab. 2. The shoot growth pat-
tern was dependent on inorganic salts and 
their concentrations. The shoot growth 
rate of P. alba was high when the plant was 
cultured in medium without NH4+ (mean 
height = 11.36 cm), while the plantlets cul-
tured in the medium free from NO3- 
showed poor shoot growth. These results 
indicate that the ammonium ion has an ad-
verse effect on plant growth. The use of ni-
trogen sources is different for each plant 
species. It has been reported that the addi-
tion of NH4+ to some plant species causes 
toxicity and reduced growth. Yan & Xu 
(2018) reported that NO3 addition signifi-
cantly stimulated the growth of above-
ground growth (8.4 g g-1 N), whereas NH4+ 
addition showed a greater effect on below-
ground growth (5.9 g g-1 N).

The plantlets cultured on the medium 
without H2PO4 showed poor shoot growth. 
Phosphorus also influenced the growth of 
P. alba. As phosphorus is an essential ma-
cronutrient for plant growth, it limits crop 
production in many regions of the world 
(Holford 1997). Gangavar & Parameshwa-
ran (1976) observed an enhancement in 
the total photosynthetic area with increasing 
levels of phosphorus, which ultimately 
produced higher amounts of dry matter.

The number of stems per individual was 
different for each medium (Tab. 3). In the 
medium with a calcium ion removed, the 
number of stems was very large. The num-
ber of leaves was also different for each 
medium. In the medium where calcium ion 
was removed, the number of leaves was 
also overwhelmingly high. There was no 
significant difference in the number of 
withered plants. The content of chlorophyll 
was significantly different among treat-
ments. The treatments with the lowest 
chlorophyll content were nitrate-free me-
dia, and there were no significant differ-
ences from the control treatments of all in-
organic salts in the media with calcium, 
magnesium and iron removed.

In the calcium-deficient medium, stem
growth was reduced, and the stem and leaf numbers increased. This result implies that calcium has a great influence on the growth of *P. alba*. Parvin et al. (2015) reported that calcium significantly increased plant height in the growth of each step of tomato. The best results here were found at a concentration of 5 mM of calcium. Calcium is a signaling molecule and acts as a second messenger which is increased in the cytosol by activating the influx channel both in the plasma membrane and tonoplast and plays a significant role in mediating mechanisms involved in the recognition and response to abiotic stresses in plants (Kader & Lindberg 2010).

The variation in biomass production was analyzed by observations with in vitro cultivated shoots after 8 weeks of culture (Tab. 4). The inorganic salt composition of the medium influenced both the above and below-ground biomass production of in vitro cultured *P. alba*. Biomass production in media with the omission of inorganic salts was enhanced compared to the control. HPO₄²⁻ and Fe³⁺ deprivation inhibited the quantity of above-ground biomass, but NH₄⁺ and K⁺ limitation enhanced the production of above-ground biomass. On the other hand, the medium without K⁺ dramatically supported below-ground biomass production. However, other treatments did not influence the production of below-ground biomass. These results indicated that phosphate is an important factor which is responsible for shoot biomass production. Fredeen et al. (1989) reported that low phosphate has the most striking effect on biomass production, with 85% reduction in total leaf area and 78% reduction in shoot dry weight at low phosphate levels in the medium. Furthermore, Fe³⁺ is known to affect plant root growth and to improve drought resistance in plants (Snyder & Schmidt 1974).

The root/shoot (R/S) ratio of *P. alba* was variable (0.09–0.29) depending upon the media components. R/S ratio was high (0.29) compared with the control (0.09) following the removal of HPO₄²⁻ from media. Also, the R/S ratio was high (1.00) in the medium free from Ca²⁺.

The chemical composition of *P. alba* biomass obtained in different media. The chemical compositions of *P. alba* grown in other media with inorganic salts removed showed significant differences (Fig. 1). Total extract contents varied between *P. alba* cultivated in media with different inorganic salts. The content of extracts (i.e., secondary metabolites) includes nonstructural components of biomass such as waxes, fats, tannins, sugars, some resins and coloring matter (Jung et al. 2010). The extractive composition of *P. alba* was 37.21% in the medium free from Ca²⁺, which was a little higher than that of *P. alba* biomass produced using control media components. This result indicated that the biosynthesis of nonstructural components such as fat and resin is independent of Ca²⁺ in *P. alba*.

Calcium treatment is known to promote plant growth and biosynthesis, as a secondary metabolite of the plant (Ahmad et al. 2016). Calcium also plays a regulatory role in plant cell metabolism, signal transduction and in the absorption of nutrients across cell membranes (Talukdar 2012).

**Lignin biosynthesis in medium revised with inorganic salts**

The lignified tissues of *P. alba* were visualized with Wiesner (phloroglucinol-HCl) reagents in 8-week-old plantlet stem middle cross-sections (Fig. 2). Although lignin was found in the whole section of *P. alba* pith, other plants grown under different nutrient conditions showed varying lignin contents from each other. Slightly later during the development of vascular tissue, cambium (ca) became covered with a continuous circumferential band of cells. The lignification appeared to extend throughout the primary medullary or interfascicular ray tissue (data not shown) and was initiated in the phloem fibers (pf) and xylem (x – Fig. 2A-H).

Among various media, the nitrogen devoid (Fig. 2B, Fig. 2C) and HPO₄⁻ removal (Fig. 2C) showed differences compared with other treatments. Lignin was noticed in the xylem tissue as well as in surround-

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**Tab. 4** Influence of mineral salts on *P. alba* biomass production. (R/S ratio): root dry weight (g) / shoot dry weight (g); (T1): Control, HSM; (T2): HSM without NH₄⁺; (T3): HSM without NO₃⁻; (T4): HSM without HPO₄²⁻; (T5): HSM without K⁺; (T6): HSM without Ca²⁺; (T7): HSM without Mg²⁺; (T8): HSM without Fe³⁺; (***): P<0.001; (**: P<0.01; (NS): not significant (P>0.05).

| Medium | Fresh Biomass (g plant⁻¹) | Dry Biomass (g plant⁻¹) | R/S ratio |
|--------|---------------------------|-------------------------|-----------|
|        | Aboveground | Underground | Aboveground | Underground | Aboveground | Underground | |
| T1     | 0.62 ± 0.01³ | 0.09 ± 0.00³ | 0.09 ± 0.00³ | 0.11 ± 0.00³ | 0.01 ± 0.00³ | 0.01 ± 0.00³ | 0.09 |
| T2     | 1.63 ± 0.06³ | 0.17 ± 0.00³ | 0.19 ± 0.03³ | 0.02 ± 0.00³ | 0.02 ± 0.00³ | 0.02 ± 0.00³ | 0.09 |
| T3     | 0.72 ± 0.01² | 0.16 ± 0.00³ | 0.12 ± 0.04³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.22 |
| T4     | 0.47 ± 0.01³ | 0.20 ± 0.00³ | 0.10 ± 0.00³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.29 |
| T5     | 1.41 ± 0.05³ | 0.19 ± 0.00³ | 0.17 ± 0.02³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.03 ± 0.00³ | 0.22 |
| T6     | 1.42 ± 0.06³ | 0.35 ± 0.00³ | 0.16 ± 0.01³ | 0.04 ± 0.00³ | 0.04 ± 0.00³ | 0.04 ± 0.00³ | 0.22 |
| T7     | 0.88 ± 0.02³ | 0.13 ± 0.00³ | 0.13 ± 0.00³ | 0.02 ± 0.00³ | 0.02 ± 0.00³ | 0.02 ± 0.00³ | 0.12 |
| T8     | 0.82 ± 0.01³ | 0.02 ± 0.00³ | 0.11 ± 0.01³ | 0.04 ± 0.00³ | 0.04 ± 0.00³ | 0.04 ± 0.00³ | 0.42 |

The root/shoot ratio is usually given as the ratio of the weight of the roots versus the top of a plant. For most trees under normal conditions, the root/shoot ratio is 1:5 to 1:6 (Kramer 1969, Perry 1982), i.e., the top is 5 to 6 times heavier than the roots (Perry 1982). The difference in the root/shoot ratio in this study is due to the relatively developed roots of the plant cultured in the medium with inorganic salts removed. An increase in soil fertility is commonly associated with a reduction in the root/shoot ratio; that is, shoot growth increases more in terms of weight than root growth (Coutts & Philipson 1980). Several investigators have reported that phosphate is a key element that strongly influences the initiation and growth of cluster roots (Shane & Lambers 2005).
Inorganic salt effect on growth and biochemical composition in Populus alba cambial tissues. In particular, the plant tissue in most test conditions showed a deep red color due to the presence of lignin, but the control and H$_2$PO$_4$-free media had less lignin. This result highlights that lignin biosynthesis acts under the strict control of H$_2$PO$_4$ contents. Mineral deficiency has been shown to affect lignin levels in crops (Frei 2013). Phosphorus deficiency has also been reported to increase lignin content (Eppendorfer & Eggum 1994). There is little information on the effect of P supply on lignin biosynthesis, but a lack or excess of inorganic elements such as P may induce the activation of enzymes involved in lignin biosynthesis (Frei 2013).

Pretreated poplar samples could be analyzed for lignin, α-cellulose, and hemicelluloses. The lignin content in P. alba was...
Fig. 3 - Fe-SEM images of α-cellulose from P. alba cultured under different nutrient conditions. (A) T1 (Control, HSM); (B) T2 (HSM without NH₄⁺); (C) T3 (HSM without NO₃⁻); (D) T4 (HSM without H₂PO₄⁻); (E) T5 (HSM without K⁺); (F) T6 (HSM without Ca⁺²); (G) T7 (HSM without Mg⁺²) and (H) T8 (HSM without Fe⁺³).

24.11% in the control and 24.78% in biomass obtained by culturing in the medium free from H₂PO₄⁻ (Fig. 1). Poplar hybrids have cellulose contents ranging from 42% to 49%, hemicellulose from 16% to 25% and total lignin contents from 21% to 29% (Sammigrahi et al. 2010). In general, the lignin content of P. alba biomass of obtained in inorganic salt-free media was higher than that of the control, except for treatment without H₂PO₄⁻. The growth of polar in the medium without H₂PO₄⁻ leads to a formation of lignin comparable to that in hybrid poplar and in other nutrient conditions. This result indicated that inorganic salts present in the medium influenced lignin biosynthesis. The high concentration of inorganic salts in the medium favors an increased cellulose level in biomass but a decreased lignin level. Entry et al. (1998) reported that a high N fertility rate decreased the concentration of lignin in longleaf pine (Pinus palustris Mill.) seedlings.

In the treatment without NH₄⁺ (T2), the lignin content was 32.57%, while the treatment without Mg⁺² (T7) showed the highest lignin content (33.14% - Fig. 1). Mg deficiency affects plant differentiation, which appears to influence lignin biosynthesis. Huang et al. (2019) also reported that Mg deficiency affected the differentiation of citrus roots. Phloem impairment in Mg-deficient leaves resulted in the cell wall lignification of both vascular cambium and spongy parenchyma cells. The hemicellulose content of P. alba was found to be 13.22-17.55% of dry weight (Fig. 1). Hemicellulose contents determined under various nutrient conditions were comparable to control plants; however, the hemicellulose content was low (13.22%) in plant material obtained from media without H₂PO₄⁻. Thus, inorganic salts slightly influenced the hemicellulose content of P. alba.

The composition of the α-cellulose present in P. alba stem is shown in Fig. 1. The α-cellulose contents of P. alba ranged between 20.74%-33.78%. Almost all cultivated plants contained a higher amount of α-cellulose than hemicellulose. However, the α-cellulose content in the tissue of popular cultivated under H₂PO₄⁻ limitation was found to be higher than others. This observation indicated that H₂PO₄⁻ is a very important inorganic salt as it favors cellulose biosynthesis in P. alba. It has often been shown that growth is correlated with lignin and cellulose concentration in stems. The cellulose content increases with the radial growth of oak trees and decreases with its height (Bodirlau et al. 2007). Accordingly, intensive lignification may stop the extension of cell walls and cause the growth cessation of plants (Miidla 1989). There is a relationship between lignin and cellulose contents; lignin and cellulose biosynthesis in plants depends on several factors related both to the plant and to the environment. Among environmental factors, nutrient exposure has a close relationship with the cellulose level in plants. Tullus et al. (2010) reported that N, P and K showed a strong relationship with lignin and cellulose levels in the stem wood of hybrid poplar clones.

The structure of α-cellulose purified from P. alba grown under control and different nutrient treatments was characterized by Fe-SEM. The morphology of the leaf sheath fibers and the cellulose microfibrils formed under differential nutrients is revealed in Fig. 3. Most of the lignin and hemicellulose was removed, and the cellulose microfibrils were separated from the original fibers on the completion of chemical treatments. The cellulose structure displayed a regular compact surface structure, and the fibers were arranged in bundles. The cellulose showed a characteristic morphology related to the nutrient growth environment. The cellulose fiber in the nutrient conditions shown in Fig. 3A, Fig. 3E, Fig. 3F and Fig. 3G showed a flat cellulose surface and did not show microfibril, whereas the cellulose surface shown in Fig. 3B exhibited both cellulose fibers and cellulose, condensed as microfibrils. Depending on the phosphate deficiency, it is necessary to look at the cellulose surface; phosphate deficiency smooths the cellulose surface. Cellulose surface morphology is related to enzyme glycosylation. In the enzymatic saccharification process for bioethanol production, the surface state and specific surface area of the substrate greatly influence the reaction rate of the enzyme with the substrate, which is one of the main factors influencing the saccharification efficiency (Youse et al. 2015). A smooth surface may prevent the enzyme solution from penetrating, thus the saccharification efficiency may be lowered (Kim et al. 2011).

Enzymatic hydrolysis and alcohol fermentation of biomass

The enzymatic digestibility of the non-de lignified raw materials was investigated with Cellulac® 1.5 L and Viscozyme L. The time course of the enzymatic hydrolysis of P. alba is shown in Fig. 4. The saccharification of biomass occurred in direct relation to time. However, the reaction rate remained relatively low after 36 h. The P. alba biomass obtained in the medium free from NO₃⁻ was saccharified to 72.0% after 36 h of hydrolysis, which was higher than for biomass produced from the treatment without NH₄⁺ (64%), without H₂PO₄⁻ (60%) and without Fe⁺³ (60%). However, the enzymatic digestibility of poplar biomass produced from Ca⁺² limited treatment was very low compared to other treatments. The maximum cellulose yield (33.78%) was obtained at T4 (free from H₂PO₄⁻), whereas the medium without NO₃⁻ (T3) showed only a 23.28% cellulose yield. However, at 100 g, the saccharification rate was 72% for T3 and 60% for T4, and when converted to sugar yield, T3 was 16.761 g and T4 was 20.268 g.
The treatment without H$_3$PO$_4$ had a higher saccharification rate than the medium without NO$_3$ because the cellulose content was high, although the saccharification rate was low.

The enzymatic hydrolysis of the biomass obtained in the phosphate-deficient medium was not high compared to other treatments. The reason for this is shown in Fig. 3. It can be stated that this may be related to the surface shape of cellulose. In other words, it is determined to be the result of the difficulty of the penetration of the enzyme solution due to the smooth cellulose surface. In addition, the difference in the saccharification rate is thought to be due to the impurities contained in the cellulose saccharification solution, the difference in the cellulose structure (length difference) and the saccharification rate. In particular, in the case of T3 and T4, the saccharification rates (72% and 60%, respectively) for 100 g of raw materials (i.e., 28% and 40%, respectively) are due to impurities. In particular, as can be seen from Fig. 1, the content of extractives is significantly higher in T4 than in other treatments. These results also suggest that inorganic salts can affect cellulose composition and therefore saccharification rates.

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). However, in this study, the enzymatic digestibility of raw materials was relatively high without pretreatments. A high hydrolysis rate was shown without pretreatment. First, it was presumed that the lignin content was low due to the lack of inorganic salt; second, the sample used for analysis was an in vitro plant. That is, it is assumed that the selective mineral limit decreases the lignin content and crystallinity of cellulose and increases the surface area.

Biomass hydrolysate was subjected to the alcoholic fermentation process. The S. cerevisiae KCCM 1215-mediated ethanol production of popular enzymatic hydrolysates is shown in Fig. 5. Ethanol from fermented enzyme hydrolysate was detected at 23 minutes of retention time (Fig. 6). Ethanol production from NO$_3$-limited hydrolysates increased and reached a maximum level after 12 h, yielding 9.58% of ethanol; however, after 24 h, ethanol production decreased. The research have reported conversions (up to 2% w/v ethanol) in 4-6 days (Spangler & Emert 1986). In this study, ethanol yielded up to 9.58% (w/v) in 12 h from cellulosics obtained from media without NO$_3$ and 9.18% (w/v) in 12 h using cellulases derived from biomass obtained from the medium without Ca$^{2+}$, respectively. The reason for promoting bioethanol production by controlling the nitrogen source is that cell wall constituents are loose. Zhang et al. (2017) also reported that nitrogen fertilizer decreased cellulosic content and lignin content.

We have yet to establish the ethanol production conditions for P. alba. Enzymatic saccharification and ethanol fermentation conditions will lead to a very high rate of ethanol productivity of 9.58%. These results indicated that the fermentation time for ethanol production could be shortened.
with plant cultivation under optimal nutrient conditions.

Inorganic salts showed both negative and positive effects on P. alba growth (Fig. 7). Among inorganic salts, H₃PO₄ is important for biomass, chemical biosynthesis and bioethanol production. Nutrient regulation may cause the unfastening of tough biomass components and help to accelerate the hydrolysis during pretreatment, which will ultimately reduce the cost of bioethanol production. An understanding of the optimal plant nutritional requirements would allow growers to adjust their fertilization program to soil types. These results can be used as basic data for proper fertilizer controls to yield quality biomass for bioethanol production in the future.

Conclusion

Our results showed that the regulation of inorganic salts is very important for biomass and bioethanol production. The appropriate use of inorganic salts can change the composition of plant cell walls. In particular, phosphoric acid and nitrogen sources have been shown to be involved in biomass production and cellulosic structure. For biomass production, H₃PO₄ showed high biomass productivity, but the enzyme saccharification rate was low, so bioethanol production from lignocellulosic biomass was found to be very important for selecting the appropriate inorganic salt.

The results of this study could be very useful for the short-term cultivation of feedstock for bioenergy production.

Author Contribution

SJS and SHY contributed equally to this paper (co-first authors).

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References

Ahmad P, Abdel Latef AA, Abd Allah EF, Hashem A, Sarwat M, Anjum NA, Gucel S (2016). Calcium and potassium supplementation enhanced growth, osmolyte secondary metabolism products, and enzymatic antioxidant machinery in cadmium-exposed chickpea (Cicer arrietum L.). Frontiers in Plant Science 7 (347): 1-10. - doi: 10.3389/fpls.2016.00313

Amichiev BY, Kurz WA, Smyth C, Van Rees KJC (2012). The carbon implications of large-scale afforestation of agriculturally marginal land with short-rotation willow in Saskatchewan. Global Change Biology Bioenergy 4: 70-87. - doi: 10.1111/j.1757-1707.2011.01100.X

Bodirau R, Spirenon I, Teaca CA (2007). Chemical investigation on wood tree species in a temperate forest in east-northern Romania. Biore- sources 2: 41-57. - doi: 10.15576/biores.2.1.41-57

Bradshaw HD, Ceulemans R, Davis J, Stettler R (2002). Emerging model systems in plant biology: poplar (Populus) as a model forest tree. Journal of Plant Growth Regulation 19: 306-313. - doi: 10.1007/s003440000030

Chapin FS (1991). Effects of multiple environmental stresses on nutrient availability and use. In: “Response of Plants to Multiple Stresses” (Mooney HA, Winner WE, Pelt JE eds). Academic Press, Cambridge, UK, pp. 67-88.

Couts MP, Philpippson JJ (1986). Mineral nutrition and tree root growth. In: “Mineral Nutrition of Fruit Trees” (Atkinson D, Jackson JE, Sharples RO, Waller WM eds). Butterworths, London, UK and Boston, MS, USA, pp. 123-136. - doi: 10.17660/Actahortic.1980.92.16

Dimitriou I, Rutz D (2015). Sustainable short rotation coppice - A handbook. Renewable Energies, Munich, Germany, pp. 104.

Entry JA, Runion GB, Prior SA, Mitchell RJ, Rogers HH (1998). Influence of CO₂ enrichment and nitrogen fertilization on tissue chemistry and carbon allocation in longleaf pine seedlings. Plant and Soil 200: 3-11. - doi: 10.1023/A: 1004305320030

Eppendorfer WH, Eggum BO (1994). Effects of sulphur, nitrogen, phosphorus, potassium, and water stress on dietary fibre fractions, starch, amino acids and on the biological value of potato protein. Plant Foods for Human Nutrition 45: 299-313. - doi: 10.1007/BF01880709

Field C, Mooney HA (1986). The photosynthesis-nitrogen relationship in wild plants. In: "On the Economy of Plant Form and Function" (Givnish TJ eds). Cambridge University Press, Cambridge, UK, pp. 25-55.

Freeden Al, Rao IM, Terry N (1989). Influence of phosphorus nutrition on growth and carbon partitioning in Glycine max. Plant Physiology 9: 229-330. - doi: 10.1000/ppl.89.3.125

Frei M (2013). Lignin characterization of a multi-faceted crop component. The Scientific World Journal 13: 1-25. - doi: 10.1155/2013/436517

Gangawar MS, Parmeshwaran PM (1976). Phosphorus and sulphur relationship in sunflower. Oilseed Journal 6: 28-32.

Geiger H, Fuggereger H (1979). Über den chemismus der Wiesner-reaktion auf lignin [On the chemistry of the Wiesner reaction on lignin]. The Zeitschrift für Naturforschung B 34: 1471-1472. [in German] - doi: 10.1515/znb-1979-1028

Holford ICR (1997). Soil phosphorus: its measurement, and its uptake by plants. Soil Research 35 (10): 59-69. - doi: 10.1071/SR9804710

Huang JH, Xu J, Ye X, Luo TY, Ren LH, Fan GC, Qi YP, Li Q, Ferrarezi RS, Chen LS (2016). Magnesium deficiency affects secondary lignification of the vascular system in Citrus sinensis seedlings. Trees 33: 171-182. - doi: 10.1007/s00468-018-1766-0

IPCC (2008). Synthesis of country progress reports. FAO, Rome, Italy.

Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C (2007). Liquefaction of lignocellulose at high-solid concentrations. Biotechnology and Bio-
