The Effect of Sucrose, Potassium, and Glutamate on the Fatty Acid Profile of *Physcomitrella patens*

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Abstract

Physcomitrella patens, a long-evolved bryophyte, produces a range of polyunsaturated fatty acids (PUFAs) through multiple desaturation and elongation processes. *P. patens* requires few resources to grow and proliferate in fatty acid production. Media engineering can effectively improve the valuable PUFAs, and here we have tested the effects of two engineered media on the changes in the fatty acid profiles of *P. patens*. A commercially available physcomitrium (cv. Gransden) was grown for 20 days at 25 °C under a 16: 8 h light (2000 Lux): dark cycle. The responses of two different media including treatment 1 with increased sucrose concentration of 30 g/L and treatment 2 composes elevated levels of sucrose (62 g/L), potassium (0.8 g/L), and glutamate (1.42 g/L) were analyzed through fatty acid profile measurements using GC-FID. Pronounced changes including an increase in capric acid elevation in treatment 1 and elevated levels in palmitic and stearic acids in treatment 2 were detected in saturated fatty acids (SFAs), highlighting the significant impact of culture medium composition. Simultaneous alterations of sucrose, potassium and glutamate levels are particularly effective in enhancing the production of SFAs. The results of these experiments are expected to provide valuable insights into optimizing fatty acid production in *P. patens*, advancing biotechnological applications, and expanding its use in industrial and nutritional fields. Future research should aim to refine nutrient concentrations, explore the molecular mechanisms of fatty acid biosynthesis, and assess environmental factors to enhance and scale up production.

Keywords: Bryophyte, Media engineering, Polyunsaturated fatty acids (PUFAs), Saturated fatty acids (SFAs), Sucrose

Introduction

Bryophyte, including mosses, liverworts, and hornworts, are among the earliest terrestrial plants, having evolved approximately 450 million years ago. Their unique adaptations to land environments are reflected in their molecular composition, particularly for lipid metabolism (Lang et al., 2018). *Phy-*

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scomitrella patens, a model bryophyte, has garnered significant attention due to its ease in genetic manipulation and available sequenced genome. In terms of sustainability, *P. patens* offers several advantages over conventional crops. It requires fewer resources, such as water and nutrients, as well as less fertile soils, making it a more environmentally friendly alternative in fatty acid production. This moss's ability to grow in diverse conditions and its lower environmental impact make it a promising candidate for producing biofuels and other eco-friendly industrial products. Unlike many other plant models, *P. patens* can be cultivated indefinitely under long-day growth conditions without spore production, allowing for extensive genetic and biochemical studies. A cultivar of this moss, 'Gransden', has been planted and studied for more than 50 years (Donoghue et al.,2021). *P. patens* has emerged as a significant bryophyte for fatty acid production, with notable implications for biotechnology and environmental sustainability. Its distinct fatty acid profiles offer unique lipid compositions that are valuable for industrial and nutritional applications.

Similar to other plants, fatty acid biosynthesis in *P. patens* begins in the plastids, where acetyl-CoA is converted into malonyl-CoA. This molecule attaches to an acyl carrier protein (ACP), a critical step in the elongation of the carbon chain by the fatty acid synthase (FAS) complex. The FAS complex adds two-carbon units from malonyl-ACP to the growing fatty acid chain, ultimately producing palmitoyl-ACP ($C_{14:0}$) and stearoyl-ACP ($C_{16:0}$) (Resemann et al., 2021). These molecules can be desaturated to form various fatty acids. The types of fatty acids produced—saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs)—play essential roles in plants and beyond in other organisms (Wells et al., 2017). Research to enhance bryophytes' fatty acids production has predominantly focused on *Marchantia polymorpha* and *P. patens*. The latter has emerged as a leading model for producing recombinant proteins and bioactive pharmaceutics, due to its high efficiency in homologous recombination and standardized cultivation techniques in photobioreactors (Horn et al., 2021). This moss is an ideal system for metabolic engineering (Zhu et al., 2021), allowing researchers to optimize biosynthetic pathways for enhanced production of targeted fatty acids. It is possible to tailor the fatty acid output of *P. patens* for various applications, ranging from industrial uses to specialized products through implementing genetic modifications.

The production of fatty acids in *P. patens* is influenced by several factors, including chemical and physical parameters such as carbon and nitrogen sources, growth factors, metal ions, temperature, pH, and aeration. Various carbon sources, including glucose, fructose, sucrose, starch, acetate, ethanol, and vegetable oils, have been shown to affect the FA production. For instance, ethanol, can be directly converted to acetyl-CoA, the starting molecule for fatty acid synthesis, providing additional reducing power for the enzymes involved in desaturation. The effect of sucrose on fatty acid composition can be attributed to its role in providing precursors for acetyl-CoA, a critical substrate for fatty acid synthesis. In plant cell cultures,

sucrose is hydrolyzed into glucose and fructose by extracellular and cell wall-bound invertase (Modarres et al., 2018). These sugars can then be converted into acetyl-CoA, which is essential for the synthesis of fatty acids. Additionally, sucrose-derived NADPH is crucial for the desaturation of fatty acids, facilitating the production of PUFAs (Kikukawa et al., 2018). Consequently, the concentration of sucrose in the culture medium directly influences the balance between saturated and unsaturated fatty acids, highlighting the need for careful optimization of sucrose levels to achieve desired fatty acid profiles. Potassium nitrate (KNO_3) plays a crucial role in maintaining high malic enzyme activity, which provides NADPH, a reducing agent necessary for fatty acid synthesis (Zhang, 2017; Zhu et al., 2018). Key parameters encompasses specific concentrations of sucrose, $CaCl_2$, and $MgSO_4$, along with precisely controlled pH and temperature (Shi et al., 2018). These findings underscore the importance of fine-tuning environmental and nutritional conditions to enhance the production of valuable fatty acids in *P. patens*, offering promising avenues for biotechnological applications and potential commercialization of these compounds. Recognizing the importance of fatty acid production, we investigated the role of media engineering on the possible changes in fatty acid profile. We implemented two treatments aimed at enhancing fatty acid yields in *P. patens*. Given the moss's potential to generate unique and valuable fatty acids, our objective was to optimize its metabolic pathways under various experimental conditions. By adjusting nutrient concentrations, such as sucrose and glutamate, we sought to identify the most effective conditions in boosting fatty acid production. This approach was designed to fully explore the potential of *P. patens* and deepen our understanding of how to maximize yields.

Material and methods

Plant material and cultivation

The Gransden cultivar of *P. patens* was obtained from the International Moss Stock Center (IMSC, Germany). The moss gametophyte was cultured on agar-containing me-

Fig. 1. The gametophyte culture of *P. patens* under various treatments (a) sucrose treatment, and (b) sucrose, potassium nitrate, and glutamate treatment

dia (Figure 1a and b), employing a basal mineral medium devoid of organic compounds. While various research groups use slightly different media formulations, the most common media for the standard growth of *P. patens* are BCD (Ashton and Cove, 1977) and Knop (Reski and Abel, 1985). Solid cultures can be initiated using inocula derived from young protonemal or gametophytic tissues. For liquid cultures, sterilized tissue samples of *P. patens* were grown in BCD medium, including 1 mM $MgSO_4$, 10 mM KNO_3, 45 µM $FeSO_4$, 1.8 mM KH_2PO_4 (pH adjusted to 6.5 with KOH), 0.22 µM $CuSO_4$, 0.19 µM $ZnSO_4$, 10 µM H_3BO_3, 0.1 µM Na_2MoO_4, 2 µM $MnCl_2$, 0.17 µM KI, and 0.23 µM $CoCl_2$, with a total pH of 5.8, supplemented with 5 ml of ammonium nitrate. The cultures were maintained at 25 °C under a 16 8 h light: dark cycle provided by fluorescent lamps with an intensity of 2000 Lux.

Treatment conditions

The experiments were conducted over 20 days to allow for medium acclimation and fatty acid profile alterations to occur. Treatment 1 contained 30 g/L sucrose to establish the baseline effect as a crucial carbon source. Treatment 2 included 62 g/L sucrose, 0.8 g/L potassium nitrate, and 1.42 g/L glutamate with some modifications. The high sucrose concentration (62 g/L) provided maximum carbon availability for fatty acid production. Potassium nitrate and glutamate were added to evaluate their roles in nitrogen metabolism and potential synergistic effects to enhance fatty acid content.

Gas chromatography

Fatty acids were extracted from the *P. patens* cells using the Folch method (Folch et al.,1957). Briefly, a solvent mixture of methanol and chloroform in a 1:2 ratio was applied in a volume twice that of the sample. Fatty acids, in the chloroform layer, were allowed to dry. To render fatty acids volatile for gas chromatography (GC), a derivatization process—specifically, methylation—was conducted according to (Kangani et al., 2008; Teng et al., 2017). Briefly, for every 10 mg of the sample (dried fatty acids), derivatization reagent, including 10 mg of KOH, 0.8 ml of methanol, and 0.2 ml of boron trifluoride-methanol was added to the reaction vessel. The mixture was incubated at 60 °C for an hour to ensure complete methylation of the fatty acids to occur. The reaction mixture was cooled, and 1 mL of each dH_2O and *n*-hexane was added to the vessel and centrifuged at 4000 rpm for 10 min for phase separation. The upper hexane layer with methylated fatty acids was carefully separated and prepared for injection into the GC. GC was performed using an Agilent gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a 100 m capillary column (Cat. No. #7489CP; Agilent) and a flame ionization detector (FID). The injector and detector temperatures were kept at 250 °C and 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.1 ml/min. Oven temperature program was 60 °C: 1 min; 24 °C at the rate of 2 °C /min; 280 °C at the rate of 10 °C /min; held isothermally for 5 min, the split ratio was 1:100. Separation and quantification of fatty acids were achieved using a standard FAME mixture containing 28 saturated and unsaturated fatty acids, including both *cis* and *trans* isomers (Cat. No. #462GLC; ChekprepNu,

Louisville, KY, USA). Peaks were identified and quantified by comparing retention times and peak areas with those of the standard mixture.

Statistical Analysis

Data analysis was performed employing a one-way ANOVA. Results were reported as mean values with corresponding standard errors (Mean ± SE). To identify significant differences between different treatment groups, a post-hoc Tukey test was applied, with significance determined at a threshold of $p \leq 0.05$.

Results

Overall, the results indicated an increase in the levels of saturated and monounsaturated fatty acids, with significant changes observed in the levels of saturated fatty acids under various treatment conditions. Increased sucrose concentration led to a significant elevation in capric acid (28%) (Figure 2). In treatment 2, a combination of sucrose, potassium nitrate, and glutamate resulted in significant increases in palmitic acid (10%) and stearic acid (12%) levels, as well as saturated fatty acid levels (Figure 3). This indicates that the synergistic effect of the compounds in treatment 2 positively influenced fatty acid metabolism.

The findings from this study highlight the importance of optimizing culture conditions to enhance the production of specific fatty acids in *P. patens* (Figure 4). The increase in SFAs and MUFAs, particularly under combined treatment conditions, suggests that manipulating the nutrient composition of the culture medium can lead to significant metabolic shifts. These shifts could be leveraged to produce higher quantities of valuable fatty acids, which have numerous applications in the pharmaceuticals, cosmetics, and food industries. Understanding the underlying mechanisms that drive these changes in fatty acid composition is essential for developing more efficient strategies for producing

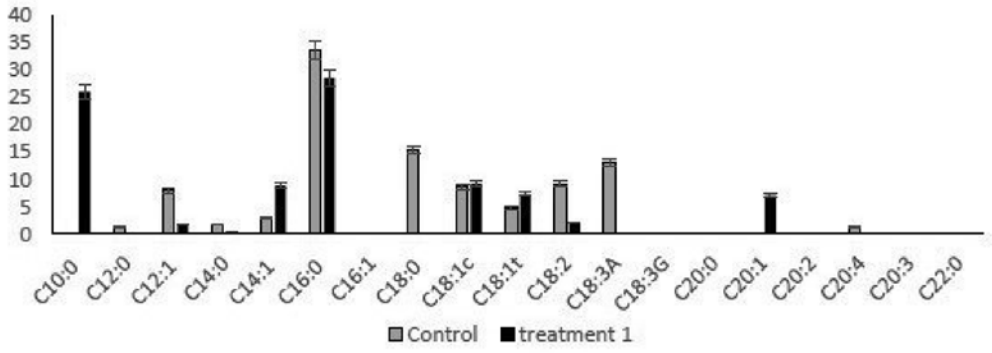

Fig. 2. The fatty acid content under sucrose treatment; all differences between control and treatments are significant at the p-value < 0.05. Fatty acids including C10:0 (capric acid), C12:0 (lauric acid), C12:1 (lauroleic acid), C14:0 (myristic acid), C14:1 (myristoleic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1c (oleic acid), C18:1t (vaccenic acid), C18:2 (linoleic acid), C18:3A (alpha linolenic acid), C18:3G (gama linolenic acid), C20:0 (arachidic acid), C20:1 (gadoleic acid), C20:2 (eicosadienoic acid), C20:4 (eicosatetraenoic acid), C20:3 (mead acid), C22:0 (behenic acid)·

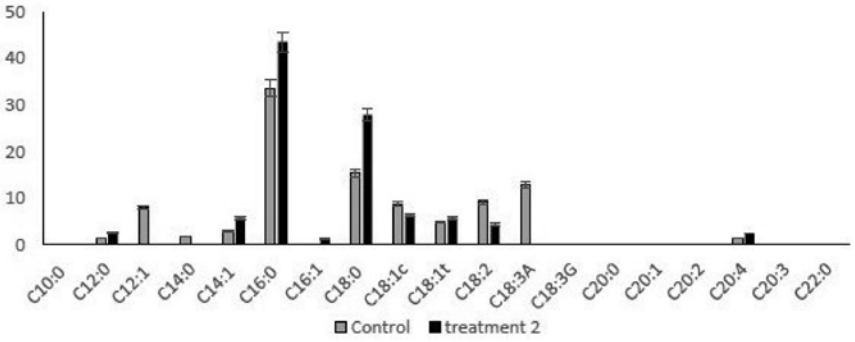

Fig. 3. Fatty acid profile of *P. patens* under combined sucrose, potassium nitrate, and glutamate treatment

Significant alterations in fatty acid composition were observed compared with the control ($p < 0.05$). Fatty acids: C10:0 (capric acid), C12:0 (lauric acid), C12:1 (lauroleic acid), C14:0 (myristic acid), C14:1 (myristoleic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1c (oleic acid), C18:1t (vaccenic acid), C18:2 (linoleic acid), C18:3A (alpha linolenic acid), C18:3G (gama linolenic acid), C20:0 (arachidic acid), C20:1 (gadoleic acid), C20:2 (eicosadienoic Acid), C20:4 (eicosatetraenoic acid), C20:3 (mead acid), C22:0 (behenic acid).

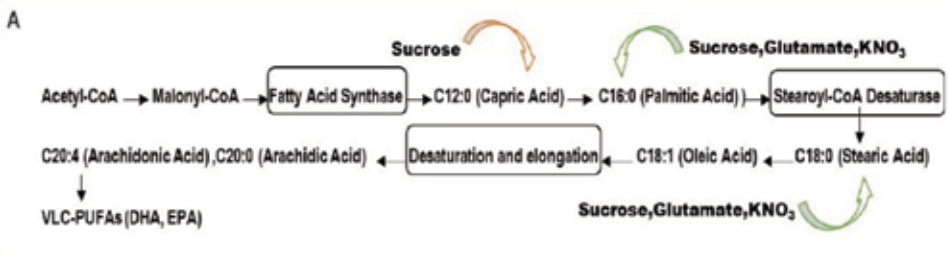

Fig. 4. Graphical workflow of this study showing (A) FA biosynthesis in *P. patens* with key enzymatic desaturation and elongation steps, (B) the life cycle, highlighting spore germination, protonema development, and formation of leafy gametophores in the haploid gametophytic phase, (C) FA extraction via the Folch method, (D) GC-FID analysis, and (E) FA composition under different treatments.

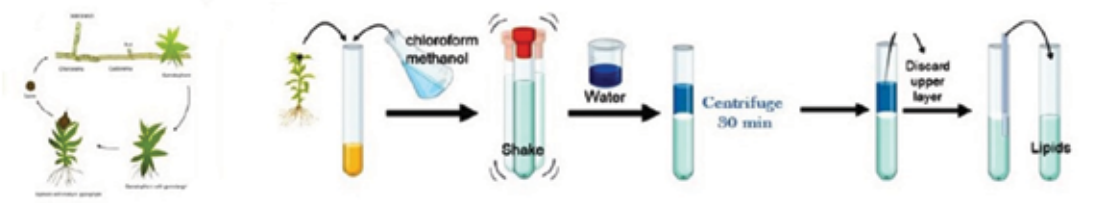

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Fig. 5. Life cycle of *P. patens* illustrating (a) the haploid gametophytic and diploid sporophytic phases, (b) overview of fatty acid extraction from *P. patens* using the Folch method

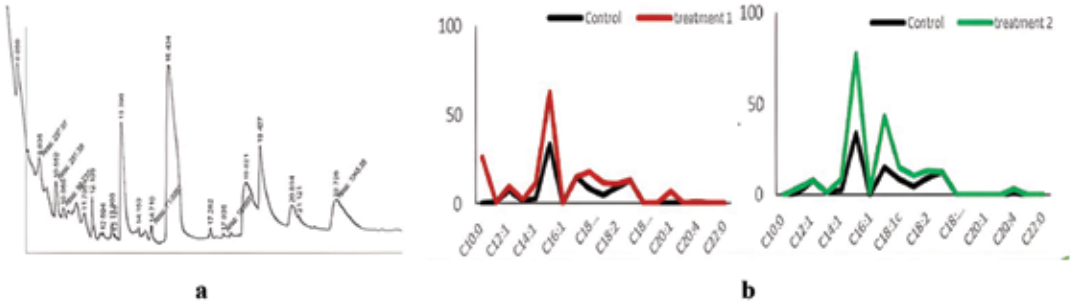

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Fig. 6. (a) Gas chromatography–flame ionization detection (GC-FID) workflow for fatty acid analysis in *P. patens*, (b) relative fatty acid composition under different treatments, showing treatment-dependent shifts in fatty acid accumulation.

high-value fatty acids in bryophytes.

Discussion

The study by Chodok (2022) aimed to develop a model to maximize the production of gamma-linolenic acid (GLA) in *P. patens* using response surface methodology (RSM). In this study, a central composite design (CCD) with three variables (sucrose, potassium nitrate, and glutamate) across five levels (-2, -1, 0, 1, 2) was employed, leading to 20 experimental runs. The resulting data were accurately fitted into a second-order polynomial model, with a coefficient of determination exceeding 0.95. Analysis of variance revealed that sucrose (20-100 g/L) and glutamate (0.5-2.5 g/L) had significant effects on GLA production. The optimized culture medium (62.92 g/L sucrose, 0.8 g/L potassium nitrate, and 1.42 g/L glutamate) increased GLA production 4.6 times compared to the standard medium after 14 days of culturing *P. patens* protoplasts in liquid medium (Chodok et al., 2022). In another study, RSM was also used to optimize biomass and adenosine deaminase (ADA) production in transgenic *P. patens*, resulting in a 3.55-fold increase in biomass and a 10.66-fold increase in ADA production (Kaewsuwan et al., 2010). Based on these studies and previous research, two different media (Treatments 1 and 2) were examined and compared with the control medium to assess the impact of sucrose concentrations and other nutrients like potassium nitrate and glutamate on the overall fatty acid content of *P. patens*.

Influence of sucrose on fatty acid composition

Sucrose, a prevalent carbon source, significantly affects the fatty acid composition in plant cultures. Studies demonstrate that higher sucrose concentrations can lead to a notable shift in fatty acid profiles. Specifically, higher sucrose levels result in a transition from a predominance of PUFAs to a greater proportion of saturated and MUFAs (Aluko et al., 2021). The optimal concentration for this shift was identified as 27% sucrose (w/v). As sucrose concentrations rise, the fatty acid profile shifts notably, with an increase in specific fatty acids such as capric acid. This suggests that sucrose functions not just as a carbon and energy source but also actively regulates fatty acid biosynthesis pathways (Hann et al, 2023). In the

presence of abundant glucose derived from sucrose, cells often prioritize glucose metabolism over the production of fatty acids. This preference can result in diminished activity of enzymes involved in the desaturation of fatty acids, such as stearoyl-CoA desaturase. As a consequence, the synthesis of saturated fatty acids and triglycerides increases, leading to elevated levels of saturated fats within the system. These findings align with observations from previous studies (Rasool et al., 2018).

When carbon sources are abundantly available, the enhanced supply of acetyl-CoA supports a more robust fatty acid synthesis process. This occurs due to a high level of carbon, which not only ensures an adequate supply of energy, in the form of ATP, to drive the biosynthetic processes but also provides the necessary precursors for the assembly of fatty acids. Consequently, the synthesis of various fatty acids, including capric acid (a medium-chain fatty acid), can be significantly increased. This is particularly crucial in metabolic pathways where specific fatty acids, here capric acid, are synthesized through the elongation of shorter carbon chains. Moreover, elevated carbon availability can positively influence the activity of key enzymes involved in the fatty acid synthesis pathway, such as acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). These enzymes play crucial roles in converting acetyl-CoA into malonyl-CoA and subsequently into longer fatty acid chains. The increased substrate availability due to high carbon levels can lead to upregulated enzyme activity, thereby boosting the overall production of fatty acids. However, the impact of carbon availability on fatty acid production is not straightforward and can vary depending on the balance and types of carbon sources present in the medium. For instance, the presence of different sugars, such as sucrose or glucose, might differently influence the metabolic flux towards fatty acid synthesis. Additionally, the activity of elongation enzymes, which are responsible for extending the carbon chains of fatty acids, is also crucial in determining the final fatty acid profile. If these elongation enzymes are not sufficiently active, even high levels of acetyl-CoA might not lead to a proportional increase in long-chain fatty acid production (Zhai et al., 2023).

Impact of glutamate on fatty acid biosynthesis

Nitrogen, primarily in the form of potassium nitrate and glutamate, significantly affects fatty acid biosynthesis, particularly the synthesis of unsaturated fatty acids. Nitrogen's role in fatty acid biosynthesis is multifaceted. Glutamate, a key nitrogen source, plays a critical role in nitrogen metabolism as a precursor for protein and nucleotide synthesis as well as a substrate for energy metabolism. It activates acetyl-CoA carboxylase (ACC), which catalyzes the formation of malonyl-CoA, an essential substrate for fatty acid synthesis and elongation. Glutamate can also be utilized directly for fatty acid synthesis through the generation of keto acids or acetyl-CoA, highlighting the importance of nitrogen in regulating the balance between saturated and unsaturated fatty acids (Sung et al., 2023).

Appropriate nitrogen levels are crucial for synthesizing desaturase enzymes, which

convert saturated fatty acids to unsaturated forms, impacting the fatty acid profile of cultured tissues. However, the impact of nitrogen on fatty acid composition is complex, as excess nitrogen can lead to negative effects (Liao et al., 2022). High concentrations of glutamate have been shown to reduce lipid and arachidonic acid (AA) production, possibly due to its conversion to proline and the associated NADPH consumption, which is essential for AA biosynthesis. Thus, while nitrogen is essential for fatty acid synthesis, its concentration must be carefully controlled to avoid negative impacts on lipid production and fatty acid composition (Zhang et al., 2022)

Nitrogen is necessary for amino acid and protein synthesis, including those involved in fatty acid metabolism. It indirectly affects capric acid production by influencing overall fatty acid synthesis and elongation pathways. Adequate nitrogen ensures the proper function of enzymes that may assist in synthesizing medium-chain fatty acids (Gebremariam and Marchetti, 2018). Nitrogen availability is crucial for palmitic acid production, as it impacts the activity of acetyl-CoA carboxylase and fatty acid synthase. Adequate nitrogen levels support effective palmitic acid synthesis. Additionally, nitrogen also affects stearic acid production, as sufficient nitrogen supports the elongation processes required to convert palmitic acid to stearic acid, thereby assisting in the maintainance of a balanced fatty acid profile.

Role of potassium in fatty acid biosynthesis

Potassium is a vital mineral that impacts plant physiology, influencing fatty acid biosynthesis and saturation. Its role is multifaceted, affecting enzyme activity, ion transport, and metabolic pathway regulation. Specifically, potassium affects the saturation levels of fatty acids in cellular membrane bilayers. Potassium acts as a cofactor for various enzymes involved in lipid metabolism. By modulating these enzymes' activities, potassium can influence the rate and direction of fatty acid synthesis and desaturation processes (Szczuko et al., 2022). Additionally, potassium is essential for maintaining membrane potential and the proper function of ion channels, which are crucial for the transport of substrates and cofactors needed for fatty acid biosynthesis across cellular membranes. Potassium also impacts signaling molecules and pathways that regulate lipid metabolism-related gene expression, altering the levels of key enzymes involved in fatty acid synthesis and modification, such as acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS) (Chen et al., 2023).

Role of multiple factors on fatty acid biosynthesis

The optimization of AA production in transgenic *P. patens* was achieved through a statistically based experimental design using response surface methodology (RSM). This approach identified that sucrose and glutamate were critical for AA production. The optimal culture conditions, determined by central composite design (CCD), were found to be 61.79 g/l sucrose, 1.18 g/l KNO_3, and 2.05 g/l glutamate, and 22.06 g/l sucrose, 1.00 g/l KNO_3, and 2.35 g/l glutamate for AA production. Under these optimized conditions (Nekrasov et al., 2019).

The oil production capabilities of the fun-

gus *Fusarium oxysporum* were analysed on various media formulations (Kozubal et al., 2019). The findings revealed that the media incorporating glucose and ammonium nitrate were most effective in maximizing oil production. Additionally, a diverse range of fatty acids with chain lengths from C8 to C24 was discovered. The fatty acid composition was found to be heavily influenced by the types of carbon and nitrogen sources, suggesting that both the quantity and quality of the oil produced are impacted by nutrient selection. In another study, the effects of phosphorus sources and nitrogen substrates on biomass production and lipid accumulation in oleaginous Mucoromycota fungi were analyzed. The study found that different nitrogen sources, such as ammonium nitrate and soybean extract, influenced fungal growth and lipid production. Additionally, varying phosphorus sources significantly impacted lipid accumulation, with certain combinations leading to increased biomass and lipid content (Dzurendova et al., 2020).

Conclusion

The intricate interplay between sucrose, nitrogen, potassium, and environmental conditions in determining fatty acid composition in plant systems underscores the importance of optimizing cultivation conditions to achieve desirable fatty acid profiles. In summary, the potential for optimizing nutrient conditions in enhancing fatty acid biosynthesis for various industrial applications in Physcomitrium was discussed. Such insights can improve the efficiency and sustainability of fatty acid production, contributing to the development of more sustainable and valuable bioproducts. The relationship between sucrose, nitrogen, and environmental conditions significantly impacts fatty acid composition in plant systems. Optimizing the factors within the culture medium is critical for achieving targeted fatty acid profiles necessary for industrial and biotechnological applications. The ability to manipulate fatty acid biosynthesis through precise nutrient and environmental management provides valuable opportunities for improving production across various sectors. Saturated fatty acids, driven by their stability, functional properties, and extended shelf life, highlight their value in areas such as food, biofuels, and materials science. However, for the production of nutrient-rich PUFAs, these treatments may not be appropriate. As research progresses, it will be crucial to develop and optimize alternative strategies that more closely align with the nutritional goals of PUFAs production, ensuring that biotechnological platforms like *P. patens* are used effectively and appropriately for the intended application.

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