Putrescine Promotes Betulin Accumulation in Suspension Cell Cultures of *Betula platyphylla* by Regulating NO and NH$_4$$^+$ Production

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Abstract: Putrescine (Put) can enhance secondary metabolite production, but its intrinsic regulatory mechanism remains unclear. In this study, Put treatment promoted betulin production and gene expression of lupeol synthase (*LUS*), one of betulin synthetic enzymes. The maximum betulin content and gene expression level of *LUS* was 4.25 mg·g^{-1} DW and 8.25 at 12 h after 1 mmol·L^{-1} Put treatment, approximately two- and four-times that in the control, respectively. Put treatment increased the content of nitric oxide (NO) and its biosynthetic enzyme activity of nitrate reductase (NR) and NO synthase (NOS). Pretreatment of the birch suspension cells with NO-specific scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline- 1-oxyl-3-oxide (cPTIO), NR inhibitor sodium azide (NaN$_3$), and NOS inhibitor N^G-nitro-L-Arg methyl ester (L-NAME) decreased Put-triggered NO generation and blocked Put-induced betulin production. Put treatment improved the content of NH$_4$$^+$ and its assimilation enzyme activity of glutamate synthase and glutamate dehydrogenase. NH$_4$$^+$ supplementation also promoted NO and betulin production. Thus, the above data indicated that Put-induced NO was essential for betulin production. NO derived from NR, NOS, and NH$_4$$^+$ mediated betulin production in birch suspension cell cultures under Put treatment.

Keywords: *Betula platyphylla*; putrescine; nitric oxide; NH$_4$$^+$; betulin

1. Introduction

Polyamines (PAs), including putrescine (Put), spermidine (Spd), and spermine (Spm), are low-molecular-weight aliphatic polycations that are quite common in living organisms [1]. Put is the major diamine in plants and a direct substrate for the synthesis of Spd and Spm [2]. PAs have been suggested to play important roles in morphogenesis, growth, embryogenesis, organ development, leaf senescence, and abiotic and biotic stress responses [3,4]. Besides the above roles, PAs also regulate various fundamental cellular processes as signaling molecules [5]. The major links in PA signaling may be hydrogen peroxide (H$_2$O$_2$) and nitric oxide (NO) [6,7].

NO is considered to cause the groundbreaking bioactive signaling described in plants [8]. Like PAs, NO also participates in various cell processes such as growth and development, respiratory metabolism, senescence, and maturation, as well as plant response to abiotic and biotic stressors [8,9]. Nitric oxide synthase (NOS) and nitrate reductase (NR) are known as two major sources of NO production in plants [10]. Moreover, NO is also produced via nonenzymatic pathways, such as NO induced by the presence of hydrogen sulfide, abscisic acid, PAs, and ammonium cation (NH$_4$$^+$) [11,12].

Recent studies have indicated that PA-induced NO biosynthesis might be a potential linking signal in many of the developmental processes and stress responses in plants. Such as, Put and Spd stimulated

arginine-dependent NO formation during dormancy release and germination of apple embryos [13]. NR is known as one of the major sources of NO production, and NO and NH_4^+ are all products of NR [14]. However, the role of NR, NO, and NH_4^+ induced by PAs in plants is poorly understood.

Plant cell culture offers a good alternative to whole-plant collection for the production of bioactive secondary metabolites under controlled and reproducible protocols. Many strategies have been used to increase the secondary metabolite production in plant cell cultures, e.g., elicitation, precursor feeding, and nutritional supplement [15,16]. One such bioactive secondary metabolite is botulin, which is extracted from the bark of white birch trees (*Betula platyphylla*); one of the pentacyclic triterpene compounds, lupeol synthase (LUS), is mainly responsible for betulin biosynthesis through the MVA (mevalonate) pathway. Betulin is a valuable metabolite with antiviral, antibacterial, and antitumor properties [17]. In our previous study, betulin was detected in birch cell suspension cultures and plantlets; PAs or NO induced betulin accumulation [18,19]. However, the regulatory mechanism of PAs or NO in betulin biosynthesis of birch suspension cells remains elusive. The aim of the present study is to determine the response of NO and NH_4^+ to Put in betulin production and investigate NH_4^+ assimilation and the sources of NO in betulin production induced by Put.

2. Results

2.1. Put Enhanced Betulin Production

To improve betulin content in birch cell suspension cultures, the induced effect of 0.1, 1, and 5 mmol·L^{-1} Put on betulin production was used for analysis. Figure 1A shows that betulin production in birch suspension cells induced by Put is time-dependent and dose-dependent. Betulin content peaked at 12 h after 0.1, 1, and 5 mmol·L^{-1} Put treatments, which were 51.89%, 139.23%, and 55.69% higher than those of the control, respectively. In addition, 5 mmol·L^{-1} Put treatment decreased betulin content at 24 and 48 h, which were 15.81% and 20.21% lower than those of the control, respectively.
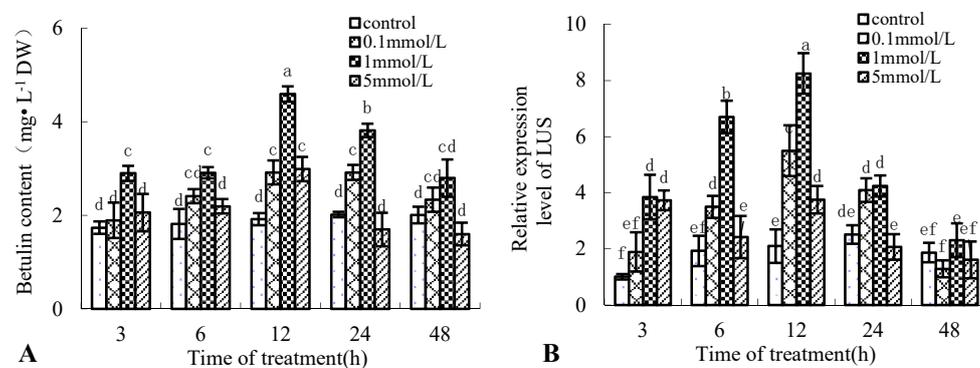
Figure 1. Effect of putrescine (Put) on betulin production and gene expression of lupeol synthase (*LUS*) in suspension cells of birch. Eight-day-old suspension cells were treated with 0.1, 1, and 5 mmol·L^{-1} Put and harvested at the indicated time points after treatment. Suspension cells treated with the same volume of distilled water were used as controls. Different letters show significant differences among means ($p < 0.05$, Tukey's test); the following figures are the same. (**A**), betulin content in suspension cells of birch; (**B**), gene expression of *LUS* in suspension cells of birch.

Betulin is a lupane-type triterpene; one of its synthetic key enzymes is lupeol synthase (LUS) [19]. Figure 1B shows that the gene expression of *LUS* has the same varying tendency with betulin content in the suspension cells of birch treated with Put; the maximum gene expression level of *LUS* was 8.25 at 12 h after 1 mmol·L^{-1} Put treatment, approximately four times that of the control.

2.2. Put Induced NO Production

NO production in birch suspension cells was examined using the Greiss reagent assay kit and a fluorometric method. Figure 2A shows the time course of NO generation in a medium of birch suspension cells treated with 0.1, 1, and 5 mmol·L^{-1} Put. NO content peaked at 9 h after 0.1 or 1 mmol·L^{-1} Put treatment, 18 h after 5 mmol·L^{-1} Put treatment, approximately 4-, 8-, and 13-times that in the control, respectively. NO fluorescence intensity in suspension cells after Put treatment had a similar change tendency to NO content in medium of birch suspension cells (Figure 2B), and the maximum fluorescence intensity was also at 9 h after 1 mmol·L^{-1} Put treatment (Figure 2C).
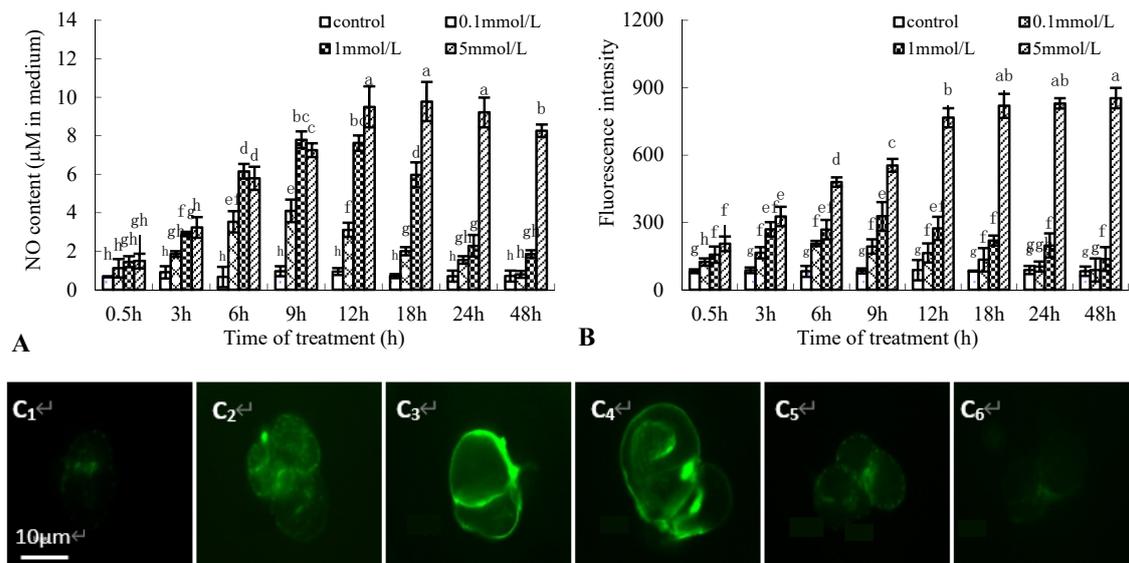
Figure 2. Effect of Put on nitric oxide (NO) generation in suspension cells of birch. (**A**), NO content in culture medium of birch; (**B**), NO fluorescence intensity in suspension cells of birch; (**C**), NO fluorescence intensity in suspension cells after 1 mmol·L^{-1} Put treatment (**C$_1$**, Control; **C$_2$**, 3 h after Put treatment; **C$_3$**, 9 h after Put treatment; **C$_4$**, 12 h after Put treatment; **C$_5$**, 24 h after Put treatment; **C$_6$**, 48 h after Put treatment).

NR and NOS are the two NO-generating key enzymes in plants [10]. Figure 3 shows that the enzyme activity of NR and NOS of the birch suspension cells induced by Put was also time-dependent and dose-dependent. Put treatment increased the enzyme activity of NR and NOS. NR enzyme activity peaked at 6 and 12 h after 5 and 1 mmol·L^{-1} Put treatment, which was 4.4-fold and 3.8-fold that of the control, respectively (Figure 3A). NOS enzyme activity peaked at 3 h after 0.1 and 1 mmol·L^{-1} Put treatment, which was 2.3-fold and 2.9-fold that of the control, respectively (Figure 3B). These results had the same change tendency as NO content in the medium of birch suspension cells.

2.3. NO-Mediated Put Induced Betulin Accumulation

To investigate the role of NO in Put-induced betulin production, we examined the effects of NO scavenger cPTIO, NOS inhibitor L-NAME, and NR inhibitor NaN$_3$ on Put-induced NO burst, betulin production, and gene expression of *LUS* in the birch suspension cells (Figure 4). As shown, pretreatment of the suspension cells with cPTIO, L-NAME, and NaN$_3$ abolished the Put-triggered NO generation (Figure 4A) and blocked the Put-induced betulin production and gene expression of *LUS* (Figure 4B,C). Thus, the data indicated that Put-induced NO was essential for betulin production, and it may have been mainly derived from NR and NOS biosynthetic pathways in birch suspension cell cultures.
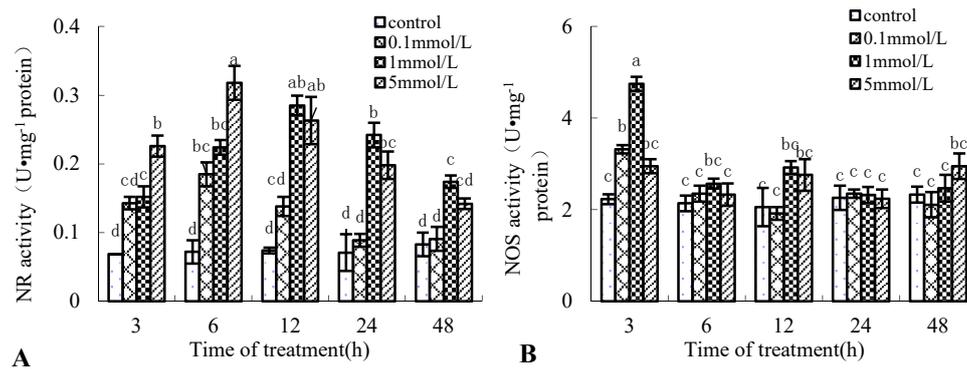
Figure 3. Effect of Put on enzyme activity of nitrate reductase (NR) and NO synthase (NOS) in suspension cells of birch. (**A**), NR activity in suspension cells of birch; (**B**), NOS activity in suspension cells of birch.
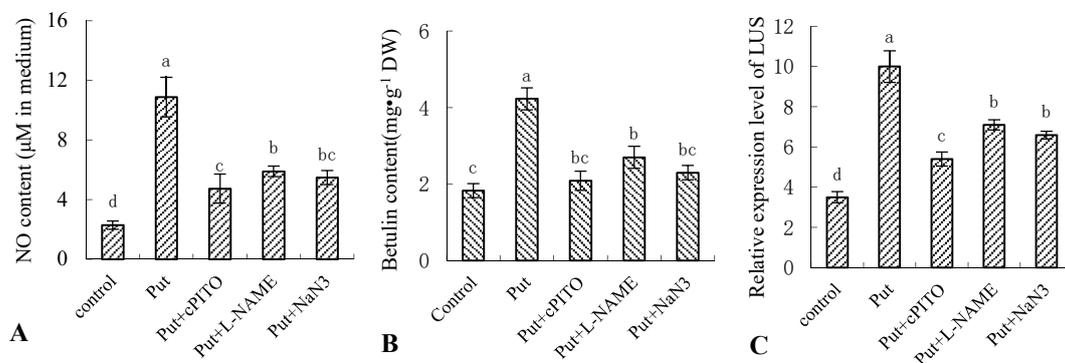
Figure 4. Effect of Put and NO inhibitor on NO production, betulin content, and gene expression of *LUS* in birch suspension cells. Eight-day-old suspension cells pretreated for 0.5 h with 150 μmol·L^{-1} 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; a NO-specific scavenger), 0.5 mmol·L^{-1} NR inhibitor sodium azide (NaN$_3$), and 3 mmol·L^{-1} NOS inhibitor N^G-nitro-L-Arg methyl ester (L-NAME) were treated with 1 mmol·L^{-1} for 12 h, respectively. (**A**), NO content in culture medium of birch; (**B**), betulin content in suspension cells of birch; (**C**), gene expression of *LUS* in suspension cells of birch.

2.4. Put Induced NH$_4^+$ Production

NO and NH$_4^+$ are all products of NR. Put treatment significantly increased the enzyme activity of NR (Figure 3A); the effect of Put treatment on NH$_4^+$ production was unknown. In this study, we examined the effect of Put treatment on NH$_4^+$ production in birch suspension cells. Figure 5A shows that Put treatment significantly increased NH$_4^+$ production except after 48 h. NH$_4^+$ content peaked at 3 h after 0.1, 1, and 5 mmol·L^{-1} Put, approximately 2.35-, 2.04-, and 2.72-fold that in the control, respectively. GS, GOGAT, and GDH are involved in ammonium assimilation [20]; the activities of GOGAT and GDH had the same change tendency as NH$_4^+$ content (Figure 5B,D), but the activity of GS was different in birch suspension cells under Put treatment (Figure 5C).

2.5. NH$_4^+$ Induced NO and Betulin Production

To further investigate the roles of NH$_4^+$-induced NO on betulin production, we examined the effect of the NO-specific scavenger cPTIO on NH$_4^+$-induced betulin accumulation in birch suspension cells. Figure 6 shows that 10 mmol·L^{-1} (NH$_4$)$_2$SO$_4$ (NS) and NH$_4$Cl (NCL) enhanced NO and betulin production, respectively. Pretreatment of birch suspension cells with cPTIO reduced NH$_4^+$-triggered NO generation (Figure 6A) and blocked NH$_4^+$-induced betulin production (Figure 6B).
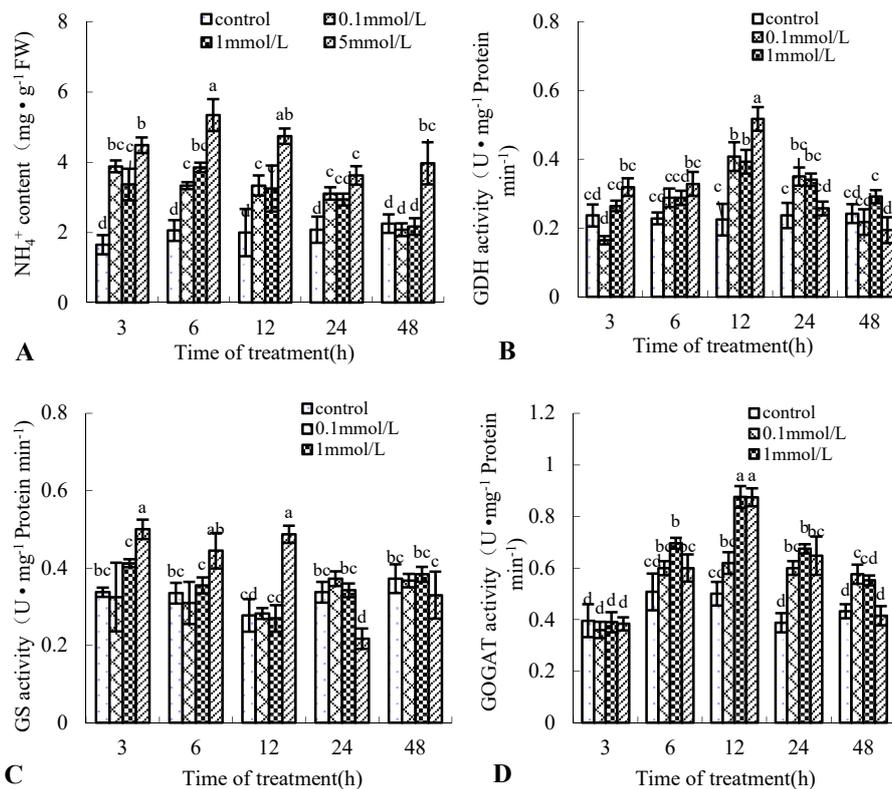
Figure 5. Effect of Put on the content of NH_4^+ and its assimilation enzyme activities in suspension cells of birch. (**A**), NH_4^+ content in culture medium of birch; (**B**), glutamate dehydrogenase (GDH) activity in suspension cells of birch; (**C**), glutamine synthase (GS) activity in suspension cells of birch; (**D**), glutamine-2-oxoglutarate aminotransferase (GOGAT) activity in suspension cells of birch.
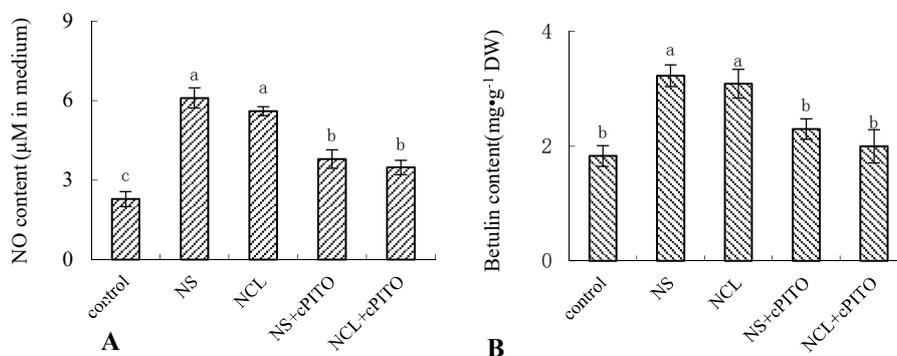
Figure 6. Effect of NH_4^+ on the content of NO and betulin in suspension cells of birch. Eight-day-old suspension cells pretreated for 0.5 h with 150 μmol·L^{-1} 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimi-dazoline-1-oxyl-3-oxide (cPTIO) (NO-specific scavenger) were treated with 10 mmol·L^{-1} (NH4)$_2$SO$_4$ (NS) and 10 mmol·L^{-1} NH$_4$Cl (NCL) for 12 h, respectively. (**A**), NO content in culture medium of birch; (**B**), betulin content in suspension cells of birch.

3. Discussion

Plants do not appear to have specific receptors for PAs; thus, it is particularly interesting to investigate how PAs perform diverse functions in plant cells [21]. Recently, some experiments have shown that Put can induce the production of NO in plants, but the roles and origins of NO induced by Put in plants remain poorly understood.

In our previous report, exogenous Put treatment increased the production of triterpenoids and flavonoids in suspension cells of birch, and exogenous NO donor sodium nitroprussiate also enhanced the accumulation of triterpenoids and the gene expression of *LUS*, one of the key enzymes in the

triterpenoid biosynthesis pathway [18,22]. Betulin is one of the main triterpenoids in the bark of birch trees. In this study, we further verified that Put treatment enhanced betulin production at the metabolic and gene expression levels and confirmed Put promoted NO production. To further clarify the function of NO-induced by Put, 8-day-old suspension cells were treated with Put and cPTIO, a specific scavenger of NO. The results showed that the Put and cPTIO treatment abolished Put-triggered NO generation and blocked Put-induced betulin production and gene expression of *LUS*. Thus, the above data suggest a crucial role of NO in Put-induced betulin accumulation in birch suspension cells.

NO is produced from a range of enzymatic and nonenzymatic sources in plants [10]. NR and NOS have been known as major enzymatic sources of NO production, although the existence of NOS in plants remains controversial [8,9]. To understand the effect of Put on NO biosynthesis, we analyzed the role of NOS and NR in Put-induced NO biosynthesis. The results showed that Put treatment enhanced the activity of NR and NOS; NR inhibitor NaN_3 or NOS inhibitor L-NAME reduced NO and betulin production in birch suspension cells induced by Put. Similar results were also reported in our previous research: NR-dependent NO and NOS-dependent NO mediated betulin production in birch suspension cells induced by a fungal elicitor or hydrogen sulfide [12,18]. Thus, our recent research suggests that NR-dependent NO and NOS-dependent NO mediate betulin production in birch suspension cells.

NO can be induced by NH_4^+ in plants [23], and NH_4^+ supply enhanced secondary metabolite biosynthesis, such as catechin and epicatechin in tea plant leaves [24,25]. NO_3^- and NH_4^+ are the two primary N sources for plants, and NR is necessary for both the first step of the reduction of NO_3^- to NH_4^+ and for the formation of NO in plants [9]. However, the role of NH_4^+-induced NO and Put -induced NH_4^+ on secondary metabolite production is unclear. Our results showed that NO scavenger cPTIO reduced NH_4^+-triggered NO generation and decreased NH_4^+-induced betulin production; Put treatment enhanced NH_4^+ production and the activities of GOGAT and GDH, which are the NH_4^+ assimilation enzymes. Awasthi et al. (2013) also found that Put treatment increased the activities of GOGAT and GDH in mice [26]. Thus, the above data first suggests that NH_4^+ or NH_4^+-triggered NO participates in Put-induced betulin accumulation in birch suspension cells, and this suggestion should be further confirmed.

The results of this work demonstrated that NO signaling was involved in Put-induced betulin production of birch suspension cells. However, the data also showed that Put could still stimulate betulin production in the birch suspension cells even though Put-triggered NO accumulation was abolished by cPTIO, which implied that Put-induced betulin production was not completely dependent on NO signaling. In addition to NO, many other signal molecules such as hydrogen peroxide (H_2O_2), jasmonic acid (JA), and salicylic acid (SA) have also been reported to play roles in secondary metabolite production [16,27,28]. Whether these signal molecules are involved in Put-induced betulin production still needs to be further investigated.

The current study allowed us to conclude that Put-induced NO is essential to betulin production in birch suspension cell cultures, and it may have been derived from NR and NOS biosynthetic pathways and nonenzymatic pathways in NH_4^+. This may be beneficial to further understanding of the role of Put-elicited betulin biosynthesis in plant cell cultures and in the rational manipulation of secondary metabolism.

4. Materials and Methods

4.1. Plant Cell Culture

The cell line used in the study was developed from the axillary buds of a 30-year-old *B. platyphylla* Suk tree. Birch suspension cell cultures were cultivated on Nagata–Takebe medium supplemented with 0.1 mg·L^{-1} 6-benzyladenine, 0.01 mg·L^{-1} thidiazuron, and 20 g·L^{-1} sucrose [29]. The medium was adjusted to pH 5.6 and then sterilized by autoclaving at 121 °C for 20 min. The suspension culture was maintained in 250 mL Erlenmeyer flasks, with a liquid volume of 100 mL in each flask, and inoculated

with 5.0 g fresh weight of 8-day-old cell suspension cultures. The Erlenmeyer flasks were incubated on a rotary shaker (110 rpm) at 25 °C. Illumination was regulated to give 14 h of light [12].

4.2. Chemical Reagents and Treatment

The chemical reagents and their concentrations used in these experiments were as follows: 0.1, 1, and 5 mmol·L^{-1} putrescine (Put), 10 mmol·L^{-1} $(NH_4)_2SO_4$, 10 mmol·L^{-1} NH_4Cl, 150 µmol·L^{-1} 2- (4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline- 1-oxyl-3-oxide (cPTIO; a NO-specific scavenger), 0.5 mmol·L^{-1} NR inhibitor sodium azide (NaN_3), and 3 mmol·L^{-1} NOS inhibitor N^G-nitro-L-Arg methyl ester (L-NAME). Among them, cPTIO was purchased from Sigma Corporation (St Louis, MO, USA); the other chemicals were purchased from Beijing Huagong Biotechnology Institute, China.

Eight-day-old suspension cells were used for the above chemical treatment. The controls were treated with the same volume of distilled water.

4.3. Betulin Estimation

Betulin was extracted from birch suspension cells using a procedure reported by Fan et al. (2014) [18]. Briefly, 0.50 g powder of dried suspension cells was soaked in 20 mL hydrochloric acid:ethanol solution (2:8 by volume). The solution was refluxed for 3 h in a water bath at 90 °C. The filtrate was extracted three times with 15 mL of ether. The combined extracts were evaporated to dryness at 40 °C and redissolved in 2 mL ethanol for HPLC analysis. Betulin content was analyzed by reversed-phase HPLC with ultraviolet detection at 210 nm. The mobile phase consisted of 80:20 (*v/v*) acetonitrile:water. The flow rate was 1 mL·min^{-1}. The betulin standard was obtained from Sigma–Aldrich Chemical Corporation (St Louis, MO, USA).

4.4. Detection of NO

Briefly, 2 mL suspension culture medium was used to determine the content of NO by the Greiss reagent method using an assay kit (Nanjing Jiangcheng Biotechnology Institute, China). The absorbance values were determined at the wavelength of 540 nm. The images of NO-induced fluorescence were examined with a confocal laser scanning microscope (Zeiss LSM 510, Mannheim, Germany) using standard filters and collection modalities for visualizing DAF-FM green fluorescence (excitation 495 nm; emission 515 nm) [30]. Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, Rockville, MD, USA) was applied to quantify the fluorescence intensity of NO; mean density = IOD SUM/area sum.

4.5. Detection of NH_4^+

Fresh suspension cells (2 g) were used to detect concentrations of NH_4^+, according to the manufacturer's instructions of commercially available assay kits (Nanjing Jiangcheng Biotechnology Institute, China). NH_4^+ was extracted in double-distilled water. The absorbance values were determined at the wavelength of 580 nm for NH_4^+ by a spectrophotometer.

4.6. Determination of Enzyme Activities

First, 2 g of fresh suspension cells was ground with liquid N_2 and then resuspended in extraction buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mmol·L^{-1} EDTA, 10% (*v/v*) glycerol, 5 mmol·L^{-1} dithiothreitol, 0.1% TritonX-100, 0.5 mmol·L^{-1} phenylmethylsulfonyl fluoride, 20 mmol·L^{-1} FAD, 1 mmol·L^{-1} leupeptin, 5 mmol·L^{-1} Na_2MoO_4, and 1% polyvinylpyrrolidone. Then, after centrifugation at 10,000×*g* for 20 min at 4 °C, the supernatant was used to determine enzyme activity.

Enzyme activities of glutamine synthase (GS), glutamine-2-oxoglutarate aminotransferase (GOGAT), glutamate dehydrogenase (GDH), and NOS were determined using commercially available assay kits (Nanjing Jiangcheng Biotechnology Institute, Nanjing, China and Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The absorbance values were recorded at the wavelength

of 540, 340, 340, and 530 nm for GS, GOGAT, GDH, and NOS by spectrophotometer, respectively. Enzyme activity was expressed as units per mg of protein.

NR activity was measured by mixing 1 volume of the extract with 5 volumes of prewarmed (25 °C) assay buffer (100 mmol·L^{-1} HEPES-KOH, pH 7.5, 5 mmol·L^{-1} KNO$_3$, and 0.25 mmol·L^{-1} NADH). The reaction was started by the addition of an assay buffer, incubated at 25 °C for 30 min, and then stopped by adding 0.1 mol·L^{-1} zinc acetate. After 15 min, the tubes were centrifuged at 13,000× *g* for 5 min. The nitrite produced was measured colorimetrically at 520 nm by adding 1 mL of 1% (*w/v*) sulfanilamide in 3 mol·L^{-1} HCl plus 1 mL of 0.02% (*v/v*) N-(1-naphthyl)-ethylenediamine in distilled water [31]. The protein content of the supernatant was determined according to the method described by Bradford (1976). Bovine serum albumin was used as a standard [32].

4.7. RNA Isolation and Real-Time Quantitative RT-PCR

Total RNA was isolated using the CTAB-based method [33]. The b-tubulin (Tu) and ubiquitin (UbQ) genes were used as references [18]. The TaqMan probes and primers are presented in Table 1. PCR amplification was performed under standard cycling conditions, namely, 94 °C for 30 s (initial polymerase activation), followed by 45 cycles of 94 °C for 12 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 40 s. Gene expression data were calculated relative to the reference gene following the $2^{-\Delta\Delta Ct}$ method [34].

Table 1. Sequences of primer pairs for quantitative real-time RT-PCR (qRT-PCR) assay.

Gene Name	Accession NO.	Primer Sequences (5′→3′)	Tm (°C)	Fragment Size (bp)
LUS	AB055511	Forward: TTGAAGACGTGCAAGAACCTG Reverse: CATCAATGAGGGATAACAAGG	58	195
TU	FG067376	Forward: TCAACCGCCTTGTCTCTCAGG Reverse:TGGCTCGAATGCACTGTTGG	54	190
UbQ	FG065618	Forward: GATTGAGGGGAGGGATGCTG Reverse: GGAGGACAAGGTGGAGGGTG	53	202

4.8. Statistical Analysis

All experiments were conducted in triplicate. Data (mean ± standard error) were statistically analyzed using SPSS version 15.0. Tukey's test was used for multiple comparisons among means, and different letters show significant differences among means ($p < 0.05$).

5. Conclusions

In this study, the application of Put significantly enhanced betulin production and gene expression of *LUS*, one of its synthetic key enzymes in birch suspension cells. Put treatment promoted NO bursts and increased the activity of NOS and NR. However, the above positive inductive effects can be blocked with NO scavenger cPTIO, NR inhibitor NaN$_3$, and NOS inhibitor L-NAME. Put treatment improved the content of NH$_4^+$ and its assimilation enzyme activity of GOGAT and GDH. NH$_4^+$ supplementation also promoted NO and betulin production. Thus, the data indicate that Put-induced NO is essential to betulin production, and NO may have been derived from NR, NOS, and NH$_4^+$ in birch suspension cell cultures induced by Put.

Author Contributions: G.F. and Y.Z. conceived and designed the experiments. Y.L. and T.Z. performed the research. G.F., Y.Z. and B.Z. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Put	putrescine
LUS	lupeol synthase
NO	nitric oxide
NR	nitrate reductase
NOS	NO synthase
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline- 1-oxyl-3-oxide
NaN_3	sodium azide
L-NAME	N^G-nitro-L-Arg methyl ester
GOGAT	glutamine-2-oxoglutarate aminotransferase
GDH	glutamate dehydrogenase
NS	$(NH_4)_2SO_4$
NCL	NH_4Cl

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