Nitric oxide is involved in the brassinolide-induced adventitious root development in cucumber (*Cucumis sativus* L.) explants

Li Yutong

Gansu Agricultural University

Yue Wu

Gansu Agricultural University

Weibiao Liao

Gansu Agricultural University

Linli Hu

Gansu Agricultural University

Mohammed Mujtaba Dawuda

Gansu Agricultural University

Xin Jin

Gansu Agricultural University

Zhongqi Tang

Gansu Agricultural University

Jianjun Yang

Gansu Agricultural University

Jihua Yu (✉ yujihuagg@163.com)

Gansu Agricultural University <https://orcid.org/0000-0001-5849-7636>

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Abstract

Background: Brassinolide (BR), as a new type of plant hormones, is involved in the process of plant growth and stress response. Previous studies have reported the roles of BR in regulating plant developmental processes and also response tolerance to abiotic stresses in plants. In my study, the main purpose is to explore whether nitric oxide (NO) is involved in the process of BR-induced adventitious root formation in cucumber (*Cucumis sativus* L.), and whether it plays a certain role.

Results: Exogenous application of 1 μM BR significantly promoted adventitious rooting, while high concentrations of BR (2-8 μM) effectively inhibited adventitious rooting. NO donor (S-nitroso-N-acetylpenicillamine, SNAP) promoted the occurrence of adventitious roots. Simultaneously, BR and SNAP mixed treatment significantly promoted adventitious rooting and the promoted effects was significantly superior to the application of BR or SNAP alone. Moreover, NO scavenger (c-PTIO) and inhibitors (L-NAME and Tungstate) inhibited the positive effects of BR on adventitious rooting. BR at 1 μM also increased endogenous NO content, NO synthase (NOS-like) and Nitrate reductase (NR) activities, while BRz (a specific BR biosynthesis inhibitor) decreased these effects. In addition, the relative expression level of *NR* was up-regulated by BR and SNAP, whereas BRz down-regulated it. The application of NO inhibitor (Tungstate) in BR also inhibited the up-regulation of *NR*.

Conclusion: BR promoted the formation of adventitious roots via inducing the generation of endogenous NO.

Background

Brassinolide (BR), a new plant hormone, was first discovered during the screening of pollen grains [1]. As a steroid hormone, it plays an important role in regulating various developmental processes, including root elongation [2] and hypocotyl and seedling growth [3]. Moreover, it mediates plant responses to various stimuli, such as chilling injury [4], salt stress [5], [heavy metal stress](#) [6] and drought stress [7]. Yuan et al. [8] reported that the alleviation role of EBL in cucumber plants under $\text{Ca}(\text{NO}_3)_2$ stress condition by regulating mineral nutrients uptake and distribution. Zhao et al. [9] demonstrated that exogenous EBR application significantly ameliorated the inhibitory effects of photosynthesis, antioxidant enzyme activity and Rubisco activase (RCA) gene expression in *Triticum aestivum* induced by a combination of drought and heat stress. The effect of BR on plant growth and development processes depends on the concentration. Low concentration of BR was suitable for callus growth and shoot regeneration in *Spartina patens*, while high concentration of epibrassinolide inhibited the growth of *Brassica* seedlings [10]. Brassinazole (BR_Z) is a specific BR biosynthesis inhibitor. BR_Z -treated cress showed dwarfism with altered leaf morphology, including the downward curling and dark green coloration of leaves [11].

Nitric oxide (NO), a ubiquitous signal molecule, plays important roles in different plant tissues and participates in a variety of physiological processes [12]. Many researchers observed that NO induced root

development in *Zea mays* [13], and it also induced seed germination, seedling development, stomatal responses, senescence, flowering and protection against pathogens in different plant species [14–19]. NO production in plants has two pathways, including enzymatic pathway and non-enzymatic pathway. Nitrate reductase (NR) and NO synthase (NOS)-like enzyme are the NO-producing enzymes identified in plants. The activities of NR and NOS-like enzymes were involved in BR signaling [20]. Moreover, as the second messenger, NO could interact with some hormones to regulate plant physiological and biochemical responses. It is involved in the signaling pathways of salicylic acid (SA), cytokinin (CTK), jasmonic acid (JA), hydrogen peroxide (H₂O₂) and indole-3-acetic acid (IAA) [21–25]. Pagnussat et al. [26] reported the role of IAA and NO in the signaling pathway during the effect of exogenous IAA on the adventitious roots of cucumber. It was clarified that NO operates downstream of IAA promoting adventitious root development through the GC-catalyzed synthesis of cGMP. Both NO and H₂O₂ played crucial roles and had synergistic effect on adventitious root development in marigold (*Tagetes erecta* L.) [27].

The formation of adventitious roots is a fundamental process of root biology, through which cells of adventitious roots form new roots after the embryo. The development of adventitious roots is a complex process regulated by various environment and plants hormones factors [28, 29]. NO mediates the auxin response leading the adventitious root formation [25]. BR-enhanced water stress tolerance in maize plants was due to BR-induced NO production [30]. BR mediated systemically NR-dependent NO generation in *Nicotiana benthamiana* [31, 32]. As mentioned above, both BR and NO could promote adventitious root development. Karpets and Kolupaev [33] reported that NO was involved in 2,4-epibrassinolide-induced heat resistance of wheat coleoptiles and in the functional interaction between NO, ROS, and calcium ions as the signal mediators. Until now, many researches have concentrated on the relationship of NO and other plant hormones [22–25]. But little is known about the relationship between BR and NO during the development of adventitious roots. In this experiment, cucumber (*Cucumis sativus* L.) was used to investigate the involvement of NO in BR-induced adventitious roots development.

Result

Effect of different concentrations of BR on adventitious root development

In order to investigate the effect of different concentrations of BR on adventitious roots, the cucumber explants were exposed to different levels of BR solutions (0, 0.2, 1, 2, 4 and 8 μ M). The root length and root number of adventitious roots first increased and then decreased with the increase of BR concentration, and both reached the maximum values at 1 μ M level (Fig. 1). Thus, the optimum concentration of BR (1 μ M) was used for further studies.

Effect of NO scavenger or inhibitors on BR induced-adventitious rooting

The effect of NO scavenger (c-PTIO), NOS-like enzyme inhibitor (L-NAME) and NR inhibitor (Tungstate) on BR-induced adventitious rooting was investigated. As shown in Fig. 2, compared with BR treatment, 200 μ M c-PTIO, 20 μ M L-NAME or 100 μ M Tungstate applied in combination with BR treatment significantly inhibited adventitious root formation. The adventitious root number and length of explants treated with SNAP (NO donor) plus BR were significantly higher than those of explants treated with either BR or SNAP alone.

Effect of BR on endogenous NO content, NOS-like and NR activity in a time-dependent manner

The time-course of NO content affected by 1 μ M BR or 1.5 μ M BR_z treatment is shown in Fig. 3A. Compared with the control, the NO content of BR-treated explants has a slow downward trend at 0h–6 h, this may be due to the wound response. From 6h to 24 h, the NO content of BR-treated explants obviously increased, subsequently, a gradual decrease appeared until 48 h (Fig. 3A). The content of NO in the BR treatment reached the maximum at 24 h and was about 1.8 times as compared to the control. In addition, the NO content of BR_z-treated explants gradually decreased from 0h to 48h, and the NO levels were always lower than those of BR-treated explant. Thus, the data suggested that BR regulated the level of endogenous NO in promoting the development of cucumber adventitious roots.

To explore the relationship between BR and NO, we further analyzed the effects of BR on the activities of NOS-like and NR enzymes in cucumber explants during rooting formation (Figs. 3B and 3C). The application of BR obviously affected the time course of NOS-like activity. The Fig. 3B illustrated that NOS-like activity in BR-treated explants decreased slightly at 0–6 h, and then increased rapidly from 6h to 24 h, and reached the maximum at 24h, which was about 2-fold of the control. In the following, from 24h to 48h, the trend of NOS-like activity changed to gradual decrease. Meanwhile, compared with the control and the BR treatment, the NOS-like enzyme activity of BR_z treatment continuously decreased throughout the whole progress (Fig. 3B). It was suggested that the inhibition of NOS-like enzyme activity by BR_z was very obvious. Similarly, as shown in Fig. 3C, the NR activity of explants treated with BR decreased transiently during the first 6 h, followed by a significant increase in NR activity from 6h to 24 h, which reached its highest activities at 24 h (about 1.4-fold of the control), and then decreased at 48 h (Fig. 3C). The NR activity of BR_z treated explants continuously decreased during the range of 0–48 h (Fig. 3C). In conclusion, the activity of the NOS and NR enzymes can be promoted by the BR treatment, and the BR_z can obviously inhibit the activity of the two enzymes. It was shown that BR regulated the production of endogenous NO by inducing the increase of NOS-like and NR enzyme activity during the adventitious root formation.

Effects of BR, SNAP, L-NAME, Tungstate and BR_z on the content of NO and BR-induced NOS-like and NR activity

In order to further verify whether NO participates in adventitious roots formation in cucumber induced by BR, the explants were placed in BR, SNAP, BR + L-NAME, BR + Tungstate and BR_z treatment for 24 h, and the content of endogenous NO and the activities of NR and NOS-like were measured. As shown in Figs. 4C, compared with the control, endogenous NO content after treatment with BR and SNAP was obviously increased by 78.03% and 84.79%, respectively. Compared with the BR treatment, when L-NAME and Tungstate were added to the BR solution, the effects of BR were reversed, NO content was reduced by 66.5% and 63.8%, respectively (Fig. 4C). Moreover, BR_z treatment alone significantly reduced NO content by 68.1% compared with the BR treatment (Fig. 4C). However, it can be seen from Figs. 4A and 4B that after treated with BR and SNAP, brighter green fluorescence was observed in the tissue position where hypocotyl produce adventitious roots, and the amount and intensity of green fluorescence are significantly higher than the control, indicating that the production of NO was also sharply rising. Compared with the distilled water treatment (CK), the BR and SNAP treatments showed obvious green fluorescence distribution. However, the BR+L-NAME, BR+ Tungstate and BR_z treatment showed that the green fluorescence in the hypocotyl was evidently lower than that in the control plants (Fig. 4A). Finally, fluorescence quantitative analysis showed that exogenous application of BR and SNAP significantly increased the production and distribution of endogenous NO in cucumber hypocotyl (Fig. 4B).

As shown in Figs. 4D and 4E, BR-induced NOS-like and NR activity were blocked by L-NAME and Tungstate. Compared with the control, application of BR and SNAP alone significantly increased the activity of NOS-like enzyme by 40.24% and 45.22%, respectively (Fig. 4D). Moreover, BR+L-NAME and BR_z treatments markedly reduced NOS-like enzyme activity by 65.92% and 66.97% compared with the BR treatment, respectively (Fig. 4D). Similarly, compared with the control, the activity of NR after treatment with BR and SNAP was obviously increased by 40.17% and 41.53%, respectively (Fig. 4E), compared with the BR treatment, the activity of NR enzyme after BR + Tungstate and BR_z treatment was drastically reduced by 41.65% and 43.59%, respectively (Fig. 4E). Thus, BR induced the generation of NO by regulating the activity of two enzymes of NOS-like and NR, and promoted adventitious roots formation in cucumber explants.

Effects of BR, BR_z, SNAP, and Tungstate on the expression of *NR*

During the adventitious rooting process, we performed real time RT-PCR to measure gene expression of *NR* (Fig. 5). Compared with the control, within 24 h after treatment, The *NR* expression levels in BR- and SNAP- treatment were significant higher than those in the control, which were 642.3% and 701.2% higher over the control (Fig. 5). There was no significant difference in the expression level of *NR* in BR + tungstate treatment. The *NR* gene expression level decreased by 89.15% and 89.69% in BR+Tungstate and BR_z treated explants compared with the BR treatment, respectively (Fig. 5). These results confirmed that BR up-regulated the expression level of *NR*, thus increasing the activity of NR enzyme, causing the increasing of endogenous NO generation, promoting the development of adventitious roots.

Discussion

BR has biological activity in the bioassay for auxin, which is similar to the function of gibberellin, ethylene and cytokinin, and it is essential for normal roots reproduction and development in plants [34, 35]. In addition, the interaction between BR and other hormones also has a certain effect on the growth and development of plants, such as, in *Arabidopsis thaliana*, BR and auxin signal transduction have interaction points, which induced the synthesis of auxin [36]; The positive effect of BR and ethylene on the reorientation stimulated by gravity has been found in *Arabidopsis thaliana* seedlings [37]; Compared with a single BL treatment, the foliar application of methyl jasmonate (MeJA) and BL in rice resulted in a remarkable reduction in infection of rice black-streaked dwarf virus (RBSDV) [38]. BR is also can participate in the activities of plant life and involved in response to various biotic and abiotic stresses. In our study, BR increased the number and length of adventitious roots in cucumber explants and the effect was dose-dependent. In earlier reports, the application of BR (0.1–10 μM) promoted hypocotyl elongation in *Arabidopsis* and the influence was related to the concentration [2, 39]. In present study, the application of 1 μM BR resulted in evident biological reaction in cucumber hypocotyl (Fig.1A and 1B). In a similar study, the application of 24-epibrassinolide (24-EBR) in excess of 1.0 μM , reduced root growth as well as root number and root length, but lower concentration of 0.1 μM EBR increased the number and root length of explants in tomato cuttings [40]. Our results also showed that BR promoted adventitious root formation which is consistent with the findings of other researchers [41–43]. Furthermore, the exogenous application of BRZ played a vital role in the inhibition of adventitious root development. The results are consistent with that of Kurepin and co-authors who also found that the application of BRZ suppressed hypocotyl growth in *Arabidopsis* and the effect depend on concentration [2]. In our experiment, exogenous BR promoted adventitious root formation, whilst BRz inhibited adventitious rooting. Some reports indicated that BR could interact with other hormones such as gibberellic acid [44], cytokinin [45] and indole acetic acid [46] to promote plant growth. In addition, some biological gaseous molecules such as carbon monoxide (CO), NO and hydrogen sulfide (H_2S) were shown to exhibit similar hormone-like effects as signal transmitters during adventitious rooting [47–49]. It has been confirmed that NO is involved in auxin response during adventitious root formation in *Arabidopsis* [50], cucumber [51], marigold [27] and *Vigna radiata* [52]. The interaction of NO with other hormones has also been extensively reported [21, 51, 53]. However, the interaction between BR and NO in adventitious root development has hardly been reported. Our results have demonstrated that BR- induced adventitious roots development was blocked by NO scavenger (C-PTIO) or inhibitors (L-NAME, Tungstate) (Fig. 2). The scavenger and inhibitors of NO not only inhibited the production of endogenous NO, but also inhibited the effect of the exogenous BR. This observation was consistent with the findings of Li et al. [52]. Our results suggested that nitric oxide was involved in the BR-induced adventitious root development in cucumber explants.

In the subsequent experiment, the involvement of endogenous NO in adventitious rooting induced by BR was further confirmed by the increased activities of NOS-like and NR (Fig. 3). BR_z reduced the production of NO and activities of NOS-like and NR, and its effect was time-dependent. Previous studies also showed that methane (CH_4) triggered the accumulation of NO in cucumber during adventitious root formation [54]. Ethephon treatments induced the increase of endogenous NO level and significantly improved

activities of NOS-like and NR during adventitious rooting [55]. Hydrogen gas induced adventitious rooting by enhancing NO level [51]. Hemin might act to promote NO accumulation in mediating adventitious root development of cucumber explants [56]. As indicated above, the endogenous accumulation of NO induced by certain exogenous substance could promote adventitious root development. Therefore, our experiment explained the fact that BR promotes the formation of adventitious root by increasing endogenous NO level and improving the activities of NOS-like and NR. Recently, it was reported that endogenous NO production and activity of NOS and NR were inhibited by c-PTIO in the eukhalophyte *Suaeda salsa* [50]. The BR induced-NO level in tea leaves was suppressed by using a NO scavenger [52] and this is consistent with our results (Fig.4). We found that exogenous application of BR and SNAP significantly increased endogenous NO production. However, L-NAME and Tungstate, BR₂ reversed the positive roles of BR and SNAP in NO accumulation. BR-induced NOS-like and NR activity was blocked by L-NAME, Tungstate and BR₂, this reduction could be recovered by BR co-treatment, similar to those reported by Tossi et al. [57]. In summary, BR induces the production of endogenous NO by improving the activity of NOS-like and NR enzymes and participates in the development of adventitious roots in cucumber. In this experiment, the endogenous NO production and distribution were clearly marked by using the DAF-FM DA fluorescent probe technology, thus verifying that BR could promote the production of endogenous NO in the process of adventitious root formation in cucumber. Moreover, NO inhibitors could reverse the role of BR in the promotion of endogenous NO, indicating that NO participated in the process of BR induced adventitious roots in cucumber. Similar studies reported that the production and distribution of endogenous NO by Green fluorescence spots were also detected in *Arabidopsis thaliana* and in maize [58, 59]. We further validated the transcript levels of NR by means of molecular biology. The data showed that the expression level of NR was up-regulated by BR and SNAP treatments. BR₂ and Tungstate treatment of NR expression was down-regulated and this reduction was recovered by BR co-treatment (Fig.5). These results are similar to that of Zhu et al. [51], who showed that 50% HRW treatment induced adventitious root development and up-regulated the expression of NR in cucumber. Xu et al. [21] also reported that ETH enhanced the relative expression of NR and improved adventitious rooting in cucumber. Therefore, BR and SNAP were verified from the transcriptional level to up-regulate the expression level of NR, thereby increasing the activity of NR enzyme, promoting the production of endogenous NO, and participating in the formation of adventitious roots.

Conclusions

In this work,our demonstrated that both BR and NO played an important role in adventitious roots formation. And BR induced adventitious roots formation via up-regulating NR gene expression and increasing the activities of NOS-like and NR enzymes, and then improving the endogenous NO level of cucumber explants, moreover, these processes depend on NO participation. That is to say, NO can participated in BR-induced adventitious root formation in cucumber explant. In our future research, the interaction mechanisms and signal transduction pathway of BR and NO will be further investigated by molecular and genetics methods.

Methods

Plant material and growth conditions

Cucumber (*Cucumis sativus* L. var. Xinchun 4) seeds were purchased from Gansu Agricultural Institute, Lanzhou, China. The seeds were surface-sterilized in 5% sodium hypochlorite for 10 min, washed with water, germinated in petri dishes with double-layer filter paper moistened with distilled water. The seeds were put in an electronic growth chamber at $25 \pm 1^\circ\text{C}$ for 5 days with a 14-h photoperiod (photosynthetically active radiation = $200 \mu\text{mol m}^{-2} \text{s}^{-1}$). Primary roots of 5-day-old seedlings were excised and then the cucumber explants were maintained under the same conditions of temperature and photoperiod for 5 days under different treatments as indicated below. The chemicals used were of analytical grade which were obtained from Chinese companies. Data were taken on root number and root length per explant.

Treatments and experimental design

Cucumber explants were placed into petri dishes (diameter = 9 cm) lined with double layer tissue paper and moistened with 60 mL distilled water as control (CK) or 60 mL of various concentration (0.2, 1, 2, 4, 8 μM) of Brassinolide (BR, Sigma, USA) and kept at $25 \pm 1^\circ\text{C}$. The following chemicals were added with suitable concentration of BR: 50 μM SNAP (S-nitroso-N-acetylpenicillamine, Sigma, United States), 200 μM c-PTIO (2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, Sigma, United States), 20 μM L-NAME (N-nitro-L-arginine methyl ester, Sigma, United States) and 100 μM Tungstate (Zhongtai Chemical Co. Ltd. Shanghai, China). Moreover, 1.5 μM BR_z (brassinazole, Sigma, United States) was administered to explants. The concentrations of these chemicals were selected based on the results of preliminary experiment conducted. The treatments were arranged in a completely randomized design in three replicates. Each experimental unit consisted of ten individual explants from which data were taken.

Determination of the endogenous NO content

The endogenous NO level was measured as described by Liao et al. [27] using the Griess reagent method. Half of gram (0.5 g) of hypocotyls (segments 1 cm long from the base of the hypocotyl) were frozen in liquid nitrogen, then ground in a mortar and homogenized in 3 mL of 50 mM ice-cold acetic acid buffer (pH 3.6, containing 4% (w/v) zinc diacetate). The homogenates were centrifuged at $10,000 \times g$ for 15 min at 4°C and the supernatants were collected.

The pellets were washed using 1.0 mL of the above extraction buffer and centrifuged. Activated charcoal (0.1 g) was added to the supernatant, which was then filtered and the absorbance was determined at 540 nm. The NO content was calculated by comparison with a standard curve of NaNO_2 .

NOS-like and NR activity determination

The activities of NR and NOS-like were analyzed by determination kits according to the manufacturer's instructions. The kits were purchased from Nanjing Jiancheng Biological Engineering Co, China.

Imaging of endogenous NO by fluorescence microscope

To obtain the image of NO production in hypocotyls, the hypocotyls (1 cm) of cucumber explants grown on plates were added 20 μ M DAF-FM DA (4-amino-5-methylamino-2,7-diamino-fluorescein diacetate, sigma) to 50 mM Tris-HCl in petri dishes in the dark for 2 h. Set the pH value at 7.5. Then, multiple washing of the Hypocotyl with distilled water to get rid of excessive fluorophore. Ten Hypocotyls under each condition were analyzed by fluorescence microscope (Leica 400x, Planapo, German Weizla). Fluorescence intensity expressed as a percentage of control was analyzed using Image J software.

Transcript level estimation with Real Time RT-PCR

Total RNA was isolated from 100 mg (fresh-weight) of excised cucumber hypocotyls (segments 1 cm long from the base of the hypocotyl) by grinding with mortar and pestle in liquid nitrogen to obtain a fine paste using TaKaRa reagent (TaKaRa Bio, Japan) according to the manufacturer's instructions. Gene-specific expression of NR for Real Time PCR were amplified using the following primers: for NR (accession number: JQ692875.1), forward 5' - AAACCCTACATCCTTCACTCTCG - 3' and reverse 5' - GGTCCATTGCCATTTCTCTTCT - 3', for actin (DQ641117), forward 5' - CCCATCTATGAGGGTTACGCC - 3' and reverse 5' - TGAGAGCATCAGTAAGGTCACGA - 3'. The total volume of each reaction is 20 μ L, contains 10 μ L SYBR Premix Ex Taq II, 2 μ L of diluted cDNA and 0.8 μ L of forward and reverse primers. PCR amplification conditions were as follows: 1 min at 95°C followed by 40 cycles of 5 s at 95°C and 20 s at 60°C with data collection at the annealing step. After the 40 cycles, we included dissociation / melting curve stage with 0 s at 95°C, 15 s at 65°C, 0 s at 95°C, subsequently with 30 s at 50°C.

Statistical analysis

Each experiment was repeated three times and the data collected were expressed as mean values $\pm$ standard error (SE). The analysis of variance was performed using SPSS Statistics 17.0 software and treatment means were separated by Duncan's multiple range test ($P < 0.05$). The cucumber seedlings were photographed after 5 days after treatment application.

Abbreviations

BR: Brassinolide; BR₂: Brassinazole; NO: Nitric oxide; SNAP: S-nitroso-N-acetylpenicillamine; c-PTIO: 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; NR: Nitrate reductase; L-NAME: N-

nitro-l-arginine methyl ester; NOS: NO synthase; 24-EBR: 24-epibrassinolide; CTK: cytokinin; JA: jasmonic acid; H₂O₂: hydrogen peroxide; IAA: indole-3-acetic acid; SA:salicylic acid;

Declarations

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Authors' contributions

YL and JY conceived and designed the research. YL and WL conducted the experiments. YL, YW, XJ, JY and ZT analyzed the data and prepared the figures and illustrations. YL wrote the manuscript. WL, LH and MD read the manuscript and made valuable inputs. All authors read and approved the submission of the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. Materials are available by contacting the corresponding author.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Effect of different concentrations of BR on the average root length and the average root number of cucumber adventitious roots development. Photographs were taken after 5 days of treatment. The different concentration of BR adventitious root length (A) and root numbers (B) were expressed as means $\pm$ SE (n = 10 explant from three independent experiments). Bars not sharing the same letters were significantly different by Duncan's multiple test ($P < 0.05$).

Figure 2

Effect of NO scavenger or inhibitors on BR induced-adventitious rooting .The primary root system was removed from hypocotyls of 5-day-old germinated cucumber. Explants were incubated with distilled water or different concentration of BR(1 μ M),SNAP 50 μ M, L-NAME(20 μ M), Tungstate(100 μ M), C-PTIO(200 μ M) as indicated for 5 days. Photographs were taken after 5 days of treatment (A). Adventitious root length (B) and root numbers (C) were expressed as means $\pm$ SE (n = 10 explant from three independent

experiments).Bars not sharing the same letters were significantly different by Duncan’s multiple test (P<0.05).

Figure 3

Effect of BR on endogenous NO content, NOS-like and NR activities in a time-dependent manner. The primary root system was removed from the hypocotyls of 5-d-old germinated cucumber seedlings. segments 1 cm long from the base of the hypocotyl as the test material .NO levels (A) of

hypocotyls(1cm) were determined by Griess reaction reagent in explants treatment with distilled water (control) or (1 μ M)BR,(1.5 μ M) BRz. NOS-like (B) and NR activities (C) of hypocotyls (1 cm)were determined using the NOS-like and NR determination Kit (Jiancheng, Nanjing, China) according to the manufacturer’s instructions in explants treatment with distilled water (control) or 1 μ M BR,1.5 μ M BRz. Values (means $\pm$ SE) are the averages of three independent experiments (n = 15 explants form each of three independent experiments),Asterisks indicate that mean values are significantly different between the treatments of BR and Control (P < 0.05) according to Duncan’s multiple test.

Figure 4

Effects of BR, SNAP, L-NAME, Tungstate and BRz on the content of NO and BR-induced NOS-like and NR activities. Effects of BR, SNAP, L-NAME, Tungstate and BRZ on the content of NO (C).The fluorescence localization of NO in cucumber explants (A),explants removed primary root were incubated with CK(distilled water), 1 μ M BR, 50 μ M SNAP, BR+20 μ M L-NAME and BR+100 μ M Tungstate, 1.5 μ M BRz for 24 h. Fluorescence intensity expressed as a percentage of control were measured using Image J analysis software(B).Then NOS-like (D) and NR (E) activity from excised cucumber hypocotyls(segments 1 cm long from the base of the hypocotyl) was analyzed using the NOS-like and NR determination Kit (Nanjing Biologic Engineering Co., China) according to the manufacturer's instructions. Mean and SE values were calculated from at least three independent experiments (n = 15). Bars denoted by the same letter did not differ significantly at P < 0.05 according to Duncan's multiple test.

Figure 5

Effects of BR, SNAP, Tungstate, BRZ on the expression of NR genes in cucumber explant. Cucumber explants were incubated with distilled water (control) or 1 μ M BR ,1.5 μ M BRZ, 50 μ M SNAP, BR+100 μ M Tungstate for 24 h, NR gene expression was analyzed by real-time (control). Bars denoted by the same letter did not differ significantly at P < 0.05 according to Duncan's multiple test.