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Nitric oxide is involved in the brassinolide-induced adventitious root development in cucumber

Yutong Li¹, Yue Wu¹, Weibiao Liao¹, Linli Hu¹, Mohammed Mujitaba Dawuda^{1,2}, Xin Jin¹, Zhongqi Tang¹, Jianjun Yang¹ and Jihua Yu^{1*} 

Abstract

Background: Brassinolide (BR), as a new type of plant hormones, is involved in the processes 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. The main purpose of our study was to explore whether nitric oxide (NO) plays a role in the process of BR-induced adventitious root formation in cucumber (*Cucumis sativus* L.).

Results: Exogenous application of 1 μ M BR significantly promoted adventitious rooting, while high concentrations of BR (2–8 μ M) effectively inhibited adventitious rooting. NO donor (S-nitroso-N-acetylpenicillamine, SNAP) promoted the occurrence of adventitious roots. Simultaneously, BR and SNAP applied together significantly promoted adventitious rooting and the combined effect was 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 μ M 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 by inducing the production of endogenous NO in cucumber.

Keywords: Brassinolide, Nitric oxide, Nitric oxide synthase, Nitrate reductase, Nitrate reductase genes

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 and hypocotyl elongation [2]. Moreover, it mediates plant responses to various stimuli, such as hypoxia stress [3], chilling injury [4], salt stress [5], heavy metal stress [6] and drought stress

[7]. Yuan et al. [8] reported that the application of 2,4-Epi-brassinolide (EBL) alleviated $\text{Ca}(\text{NO}_3)_2$ stress in cucumber plants by regulating mineral nutrients uptake and distribution. Zhao et al. [9] also found that exogenous EBR application 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* [10], while high concentration of epibrassinolide inhibited

* Correspondence: yujihuagg@163.com

¹College of Horticulture, Gansu Agricultural University, Lanzhou 730070, People's Republic of China

Full list of author information is available at the end of the article



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the growth of *Brassica oleraceae* cotyledons [11]. 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 color typical of *Arabidopsis* BR-deficient mutants and the application of 10 nM brassinolide could reverse the dwarfism [12].

Nitric oxide (NO), a ubiquitous signal molecule, plays important roles in different plant tissues and participates in a variety of physiological processes [13]. Many researchers observed that NO induced root development in *Zea mays* [14], and it also induced seed germination, seedling development, stomatal responses, senescence, flowering and protection against pathogens in different plant species [15–20]. 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 [21]. Zhu et al. [22] have reported that NO production through NOS and NR pathways was involved in adventitious rooting of cucumber explants induced by H₂. The activities of NR and NOS-like enzymes were involved in BR signaling [23]. 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), ethylene (ETH), hydrogen peroxide (H₂O₂) and indole-3-acetic acid (IAA) [24–28]. Pagnussat et al. [29] 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.) [30].

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 [31, 32]. Pagnussat et al. [27] observed that a transient increase in NO concentration was required and was part of the molecular events involved in adventitious root development induced by indole acetic acid (IAA), indicating that NO mediates the auxin response leading the adventitious root formation. BR-enhanced water stress tolerance in maize plants was due to BR-induced NO production and NO-activated ABA biosynthesis [33]. The existence of a signaling pathway leading to BR-mediated systemic virus resistance involves local Respiratory Burst Oxidase Homolog B (RBOHB)-dependent H₂O₂ production and subsequent systemic NR-dependent NO generation [34]. Kwak et al. [35] reported that lower concentrations of BL increased

the number and length of adventitious roots while higher concentrations of BL caused trichome-like roots. As mentioned above, both BR and NO could promote adventitious root development, which suggest a possible relationship between BR and NO. Karpets and Kolupaev [36] reported that NO was involved in 2,4-epibrassinolide-induced heat resistance of wheat coleoptiles and the functional interaction between NO, ROS, and calcium ions as the signal mediators. Until now, many researches focused on studying the relationship between NO and other plant hormones [24–27]. However, little is known about the relationship between BR and NO during the development of adventitious roots. To explore this issue, pharmacological experiments were conducted using cucumber (*Cucumis sativus* L.) as test material to investigate the role of NO in BR-induced adventitious roots development. The results provide new insights into the involvement of NO in BR-induced adventitious roots development in cucumber.

Result

BR concentrations affect number and length of adventitious roots

In order to investigate the effect of BR on adventitious roots, the cucumber explants were exposed to different concentrations of BR (0, 0.2, 1, 2, 4 and 8 μM). The root length and root number of adventitious roots initially increased and then decreased with the increase of BR concentration, both reaching the maximum values at 1 μM (Fig. 1). Thus, the optimum concentration of BR (1 μM) was used in the subsequent experiment.

Number and length of adventitious root are affected by NO scavenger and inhibitors

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 μM c-PTIO, 20 μM L-NAME or 100 μM 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.

Temporal regulation of endogenous NO content, NOS-like and NR activity by BR

The time-course of NO content as affected by 1 μM BR or 1.5 μM 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 0 h–6 h, which may be due to the wound response. From 6 h to 24 h, the NO content of BR-treated explants increased, and subsequently gradually decreased until 48 h (Fig. 3a). The content of NO in the BR treatment reached the maximum at 24 h and was about

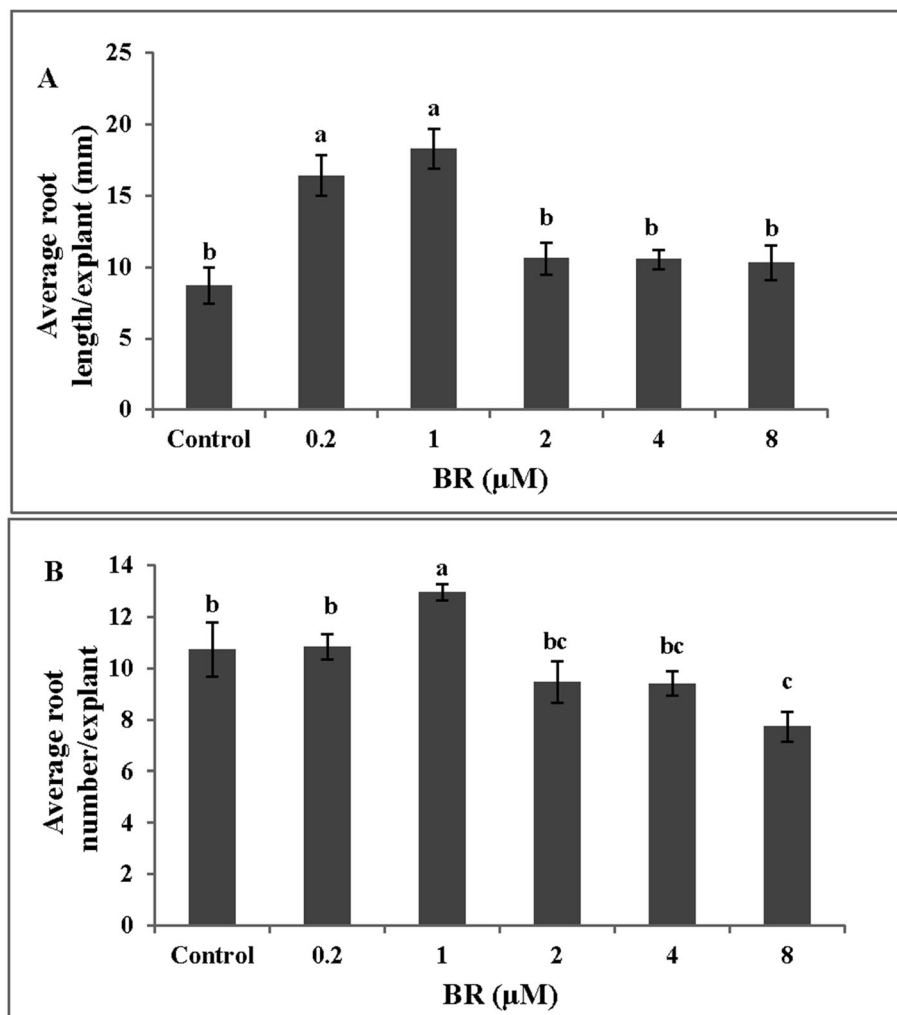


Fig. 1 Effect of different concentrations of BR on the average root length and the average root number of cucumber adventitious roots development. Photographs were taken after 5 days of treatment. The different concentration of BR adventitious root length (**a**) and root numbers (**b**) were expressed as means \pm SE ($n = 10$ explants from three independent experiments). Bars denoted by different letters were significantly different according to Duncan's multiple test ($P < 0.05$)

1.8 times as compared to the control. In addition, the NO content of BRz-treated explants gradually decreased from 0 h to 48 h, and the NO levels was always lower than those of BR-treated explant. Thus, the data suggest that BR regulated endogenous NO to promote the development of adventitious roots in cucumber.

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 the formation of adventitious roots (Fig. 3b and c). The application of BR distinctly affected the time course of NOS-like activity. The NOS-like activity in BR-treated explants decreased slightly at 0–6 h, and then increased from 6 h to 24 h, reaching a maximum at 24 h, which was about 2-fold of the control. Then, from 24 h to 48 h, NOS-like activity tended to gradually decreased (Fig.

3b). Meanwhile, compared with the control and the BR treatment, the NOS-like enzyme activity of BRz treatment consistently decreased throughout the whole experiment (Fig. 3b). Similarly, the NR activity of explants treated with BR decreased transiently during the first 6 h period, followed by a significant increase from 6 h 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). However, the NR activity in BRz-treated explants continuously decreased during the time of experiment (Fig. 3c). In conclusion, the activity of NOS-like and NR enzymes were promoted by BR treatment, while the BRz inhibited the activities of these two enzymes. Here, we showed that BR regulated the production of endogenous NO by inducing the increase activity of NOS-like and NR enzyme during the adventitious root formation.

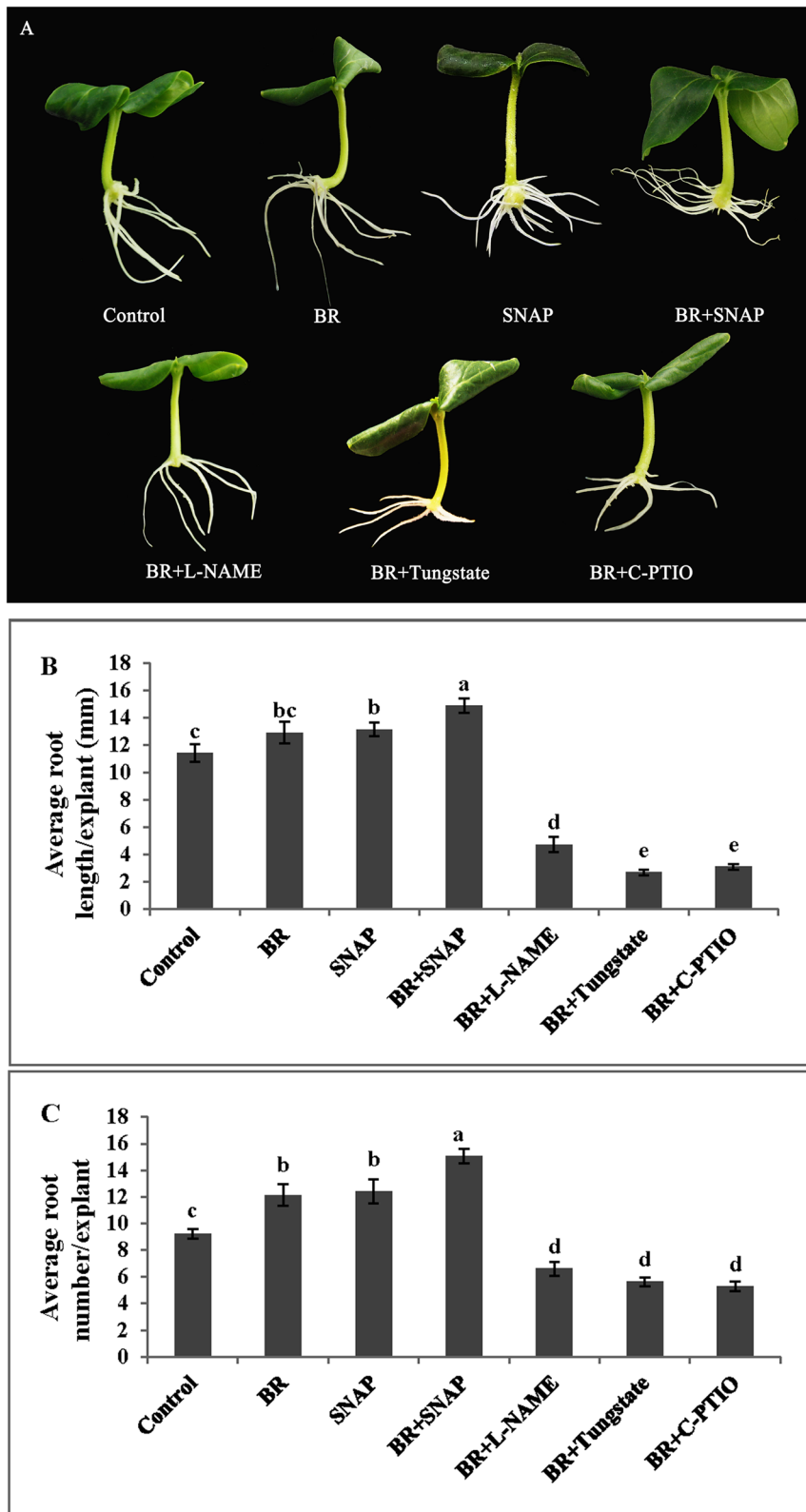


Fig. 2 (See legend on next page.)

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Fig. 2 Effect of NO scavenger or inhibitors on BR-induced adventitious root formation. The primary root was removed from hypocotyls of 5-day-old germinated cucumber seedling. Explants were incubated in distilled water, 1 μ M BR, 50 μ M SNAP, 20 μ M L-NAME, 100 μ M Tungstate, and 200 μ M c-PTIO for 5 days. Photographs were taken 5 days after treatment (a). Adventitious root length (b) and root numbers (c) were expressed as means \pm SE ($n = 10$ explants from three independent experiment). Bars denoted by different letters were significantly different according to Duncan's multiple test ($P < 0.05$)

NO content, NOS-like and NR activity under BR, SNAP, L-NAME, tungstate and BR_Z treatments

In order to further verify whether NO participates in BR-induced adventitious roots formation in cucumber, the explants were placed in BR, SNAP, BR + L-NAME, BR + Tungstate and BR_Z treatment for 24 h. The fluorescence localization of NO in hypocotyl, the content of endogenous NO and the activities of NR and NOS-like enzymes were analyzed. As shown in Fig. 4a and b, after treatment with BR and SNAP, brighter green fluorescence was observed in the tissue at the place where hypocotyl produce adventitious roots, and the intensity of fluorescence was significantly higher than in control explants, indicating that the production of NO was sharply rising. In opposite, explants treated with BR + L-NAME, BR + Tungstate and BR_Z showed a lower fluorescence in the hypocotyl than in the control plants (Fig. 4a and b). In order to support the qualitative analysis, the quantification of endogenous NO content was done in hypocotyl of cucumber explants. As shown in Figs. 4c, compared with the control, endogenous NO content after treatment with BR and SNAP was significantly 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. Indeed, 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). The qualitative and quantitative analyses of NO in hypocotyl of cucumber explants showed that exogenous application of BR and SNAP significantly increased the production and distribution of endogenous NO in cucumber hypocotyl. As shown in Fig. 4d and e, 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 BR and SNAP treatment was significantly 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 NOS-like and NR enzymes, and promoted adventitious root formation in cucumber explants.

The relative expression of NR gene under BR, BR_Z, SNAP, and tungstate treatments

During the adventitious rooting process, we performed real time RT-PCR to measure the relative expression of NR gene (Fig. 5). Compared with the control, the NR expression levels in BR- and SNAP- treatment were significantly higher than those in the control at 24 h after treatment, which were 642.3 and 701.2% higher over in the control (Fig. 5). There was no significant difference in the relative expression level of NR between BR + tungstate and the control. The relative expression level of NR gene decreased by 89.15 and 89.69% in BR + Tungstate and BR_Z treated explants compared with the BR treatment, respectively (Fig. 5).

Discussion

The data reported here demonstrated the interaction between BR and NO during adventitious rooting in cucumber explants. The results showed that exogenous BR enhanced the number and length of adventitious root at low concentrations, while high concentrations of BR treatments suppressed adventitious root development (Fig. 1), which suggested that the effect of BR on adventitious root is a dose-dependent response. Previous studies showed that the application of BR (0.1–10 μ M) promoted hypocotyl elongation in *Arabidopsis* and the influence was related to the concentrations [2, 38]. Similarly, in tomato, Guan and Roddick [39] reported that the application of 24-epibrassinolide (24-EBR) in excess of 1.0 μ M reduced root growth as well as root number and root length, but lower concentration of 0.1 μ M EBR increased the number and root length. It may be noted that the optimum concentration of BR for rooting in previous research [2, 38] was different from our experiment. It might be due to the sensitivity to BR of the different plant species.

BR has biological activity in the bioassay for auxin, which is similar to the function of gibberellin, ethylene and cytokinin, and it affects root formation and development in plants [40, 41]. BR also participates in the plant life processes, such as responses to various biotic and abiotic stresses [42–44]. Our results also showed that BR promoted adventitious root formation. Furthermore, the

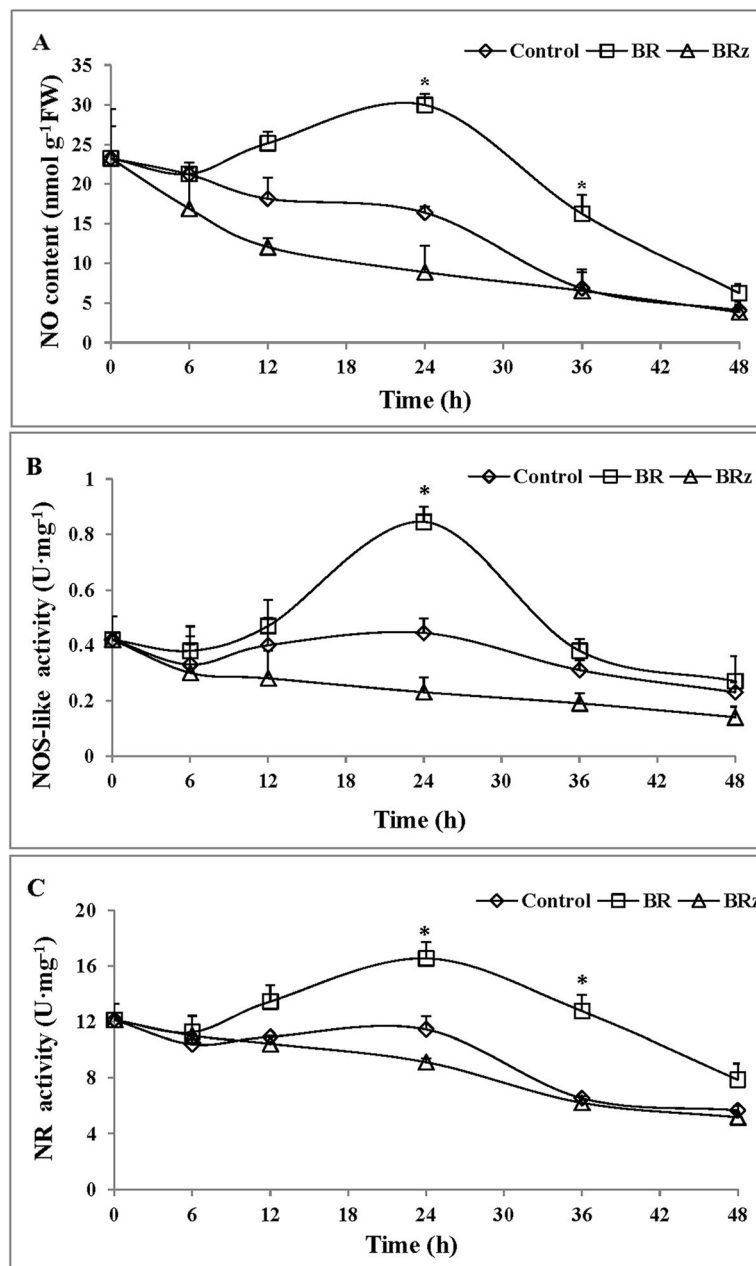


Fig. 3 Effect of BR on endogenous NO content, NOS-like and NR activities in a time- dependent manner. The primary root system was removed from the hypocotyls of 5-d-old germinated cucumber seedlings. Enzymatic activities were measured from 1 cm-long segment taken from the base of hypocotyl. NO levels (a) were determined by Griess reaction reagent in hypocotyls of explants treated 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 treated 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 from each of three independent experiments). Asterisks indicate that mean values are significantly different between treatments and control ($P < 0.05$) according to Duncan's multiple test

exogenous application of BR_Z 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 BR_Z suppressed hypocotyl growth in *Arabidopsis* and the effect depended on the concentration applied [2]. In addition, the interaction

between BR and other hormones also has a certain effect on the growth and development of plants [45–47]. For example, in *Arabidopsis thaliana*, BR and auxin signal transduction have interaction points, which induced the synthesis of auxin [48]. The interaction of Brassinolide and ethylene can control the negative gravitropism of

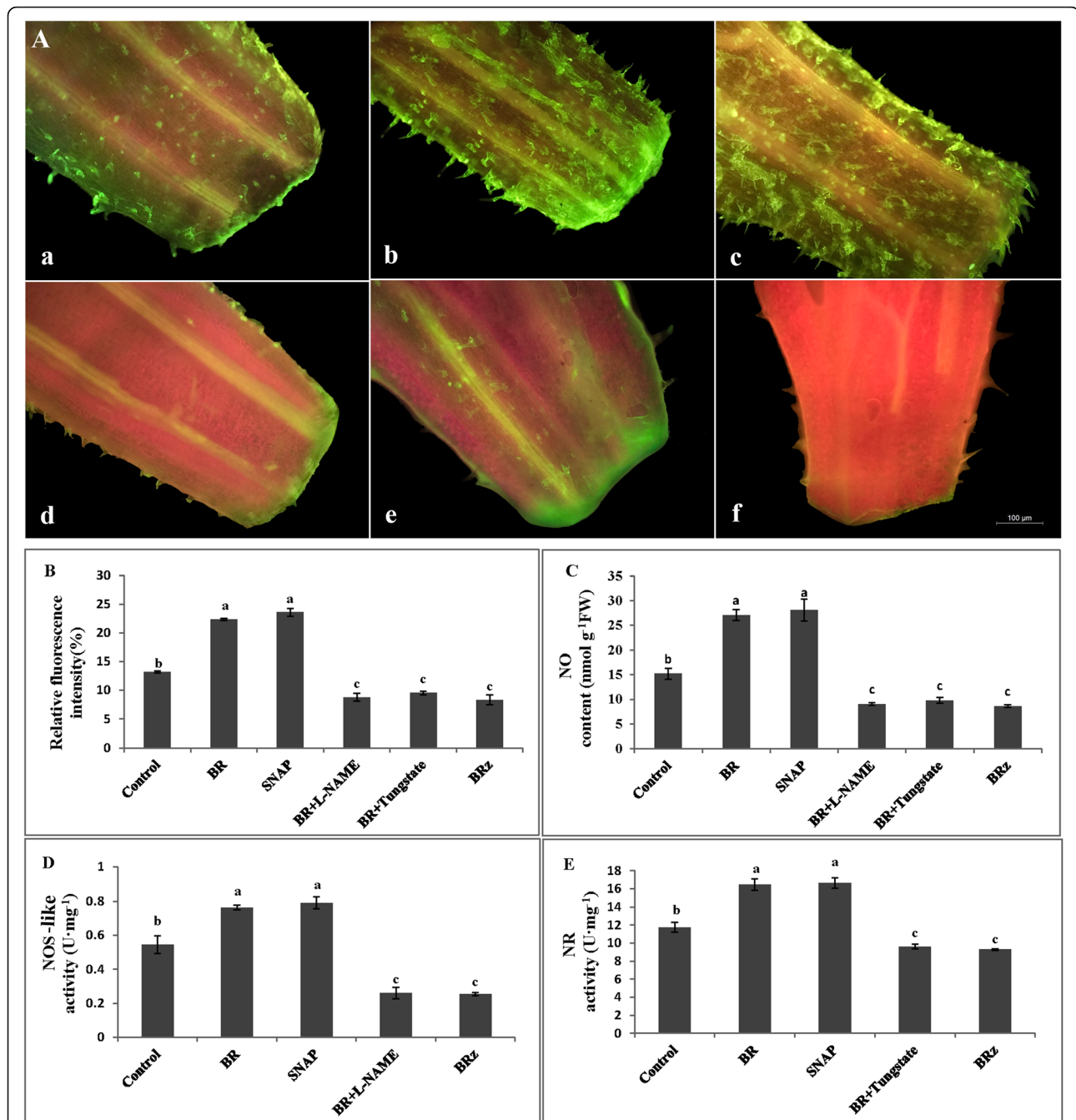


Fig. 4 Effects of BR, SNAP, L-NAME, Tungstate and BRz on the content of NO and BR-induced NOS-like and NR activities. The fluorescence localization of NO in hypocotyl of cucumber explants (A) treated with control (distilled water) (a), 1 μ M BR(b), 50 μ M SNAP (c), BR + 20 μ M L-NAME (d) and BR + 100 μ M Tungstate (e), 1.5 μ M BRz (f) for 24 h. Fluorescence intensity was analyzed using Image J software and expressed as a percentage of the control (B). The quantification of endogenous NO content was done in hypocotyl of cucumber explants (C). NOS-like (D) and NR (E) enzymatic activities were measured from 1 cm-long segment taken from the base of hypocotyl. Mean and SE values were calculated from 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

Arabidopsis seedlings, and it depends on the signal component of auxin [49]. Compared with a single epibrassinolide (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) [50]. Moreover, some biological gaseous molecules such as carbon monoxide (CO), NO and hydrogen peroxide (H_2O_2) were shown to exhibit similar hormone-like effects as signal transmitters during adventitious rooting [51–53]. It has been confirmed that NO is involved in

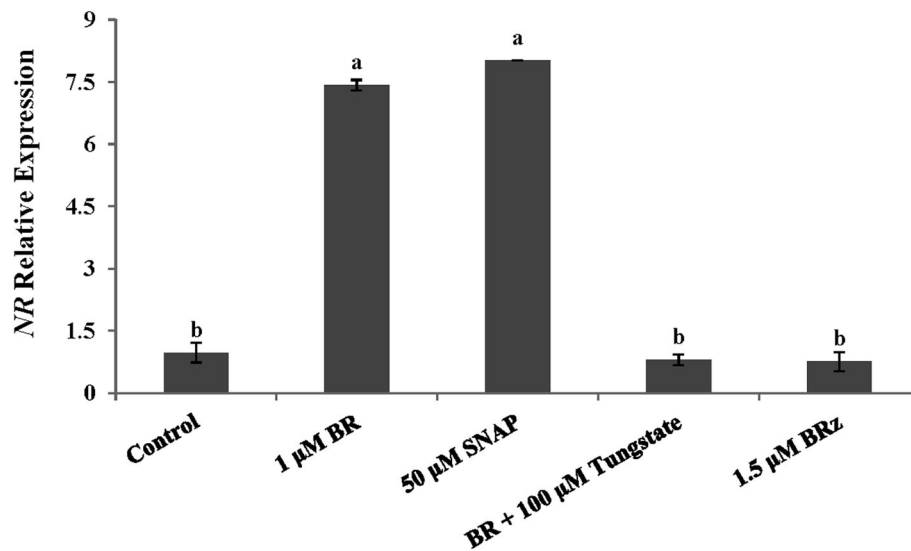


Fig. 5 Analysis by qRT-PCR of the effect of BR, SNAP, tungstate and BRz on the expression of *NR* gene in cucumber explants. Explants were incubated for 24 h in water (control) or in the presence of the different compounds in concentration as indicated on the figure. Relative expression was determined using the $\Delta\Delta$ Ct method [37], taking *actin* gene as a reference. Data were collected from 3 independent biological replicates. Each replicate represents a pool of ten explants. Bars denoted by identical letters did not differ significantly (Duncan's multiple test; $P < 0.05$)

auxin induced adventitious root formation in marigold [30]. The interaction of NO with other hormones has also been extensively reported [22, 54, 55]. 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 inhibited 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. [56]. Our results suggested that nitric oxide was involved in the brassinolide-induced adventitious root development in cucumber explants.

In the subsequent experiment, the involvement of endogenous NO in BR-induced adventitious rooting was further confirmed by the increased activities of NOS-like and NR (Fig. 3). BRz 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 [57]. Ethephon treatments induced the increase of endogenous NO level and significantly improved activities of NOS-like and NR during adventitious rooting [58]. Hydrogen gas induced adventitious rooting by enhancing NO level [22]. Hemin might act to promote NO accumulation in mediating adventitious root development of cucumber explants [59]. 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 enzymes. Recently, it was reported that endogenous NO production and activity of NOS-like and NR were inhibited by c-PTIO in the euhalophyte *Suaeda salsa* [60]. The NO content in tea leaves induced by BR was suppressed when using a NO scavenger [54]. This latest observation was 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, BRz reversed the positive roles of BR and SNAP in NO accumulation. The induction of NOS-like and NR activities induced by BR were inhibited by L-NAME, Tungstate and BRz. In summary, BR induced the production of endogenous NO by stimulating the activity of NOS-like and NR enzymes and promoted in the development of adventitious roots in cucumber. In the present study, the endogenous NO production and distribution were visualized in planta by the DAF-FM DA fluorescent probe technology, thus verifying that BR could promote the production and accumulation of endogenous NO during adventitious rooting 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 were also detected by DAF-2DA, a fluorescent NO indicator dye in *Arabidopsis thaliana* and in maize [33, 61]. Indeed, it is known that NR activity on

one hand is regulated by phosphorylation and interaction with 14-3-3 proteins [62, 63], on the other hand, the activity of NR is affected by the abundance of NR enzyme in a certain degree. Whereas the abundance of the NR protein is affected by the accumulation of the NR gene transcript. Therefore, we further investigated the regulation of NR gene expression. Our data showed that both BR and SNAP induced the accumulation of the transcript, whereas BR_Z and Tungstate treatment down-regulated the relative expression level of NR (Fig. 5). These results are similar to that of Zhu et al. [22], who showed that 50% hydrogen-rich water (HRW) treatment induced adventitious root development and up-regulated the expression of NR in cucumber. Xu et al. [54] also reported that ETH enhanced the relative expression of NR and improved adventitious rooting in cucumber. Taken altogether, the data strongly suggest that BR and SNAP stimulate the activity of NR enzyme, inducing the accumulation of transcripts and probably the corresponding protein, causing the production and accumulation of NO and promoting, subsequently, adventitious rooting in cucumber. Similarly, in cucumber, Zhu et al. [22] have reported that nitric oxide is required for hydrogen gas-induced adventitious root formation and Pagnussat et al. [29] have reported that nitric oxide and cyclic GMP are involved in the indole acetic acid-induced adventitious rooting process, which suggests that hydrogen gas and indole acetic acid might be the upstream signal molecule of nitric oxide during adventitious rooting process. It will provide us a new research topic about the up-and-downstream relationship of BR, hydrogen gas and indole acetic acid on adventitious root formation.

Conclusions

In the present study, we demonstrated that both BR and NO played important role during adventitious roots formation in cucumber explants. BR induced adventitious roots formation by up-regulating the relative expression level of NR gene and increasing the activities of NOS-like and NR enzymes, and then improving the endogenous NO level of cucumber explants. In this regard, we argue that NO is involved 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 investigated by molecular and genetics methods.

Methods

Plant material and growth conditions

The seeds of cucumber, *Cucumis sativus* L. var. Xinchun 4, were purchased from Gansu Academy of Agricultural Sciences (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 analytical grade chemicals used in the study were obtained from Chinese companies. Root number and root length per explant were recorded and analyzed.

Treatments and experimental design

Cucumber explants were placed in petri dishes (diameter = 9 cm) lined with double layer tissue paper and moistened with 60 mL distilled water as control (control) 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 experiments (data not shown). 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. Data are expressed as means \pm standard error (SE).

Determination of root number and length of cucumber explants

Five days after treatments, the root number of each explant was counted and the root length of each explant was measured with a ruler (accuracy is 0.1 cm). Three independent biological replications were done and ten explants of each replication were analyzed. Data were expressed as the average \pm SE.

Determination of the endogenous NO content

The endogenous NO level was measured as described by Liao et al. [30] 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 with mortar and pestle, 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×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 NaNO₂. Measurements were done in 3 independent biological replicates and data represent the average ± SE.

NOS-like and NR activity determination

The activities of NR and NOS-like were analyzed by nitrate reductase assay kit and nitric oxide synthase assay kit according to the manufacturer's instructions. The kits were purchased from Nanjing Jiancheng Biological Engineering Co, China. Measurements were done in 3 independent biological replicates and data represent the average ± SE.

Imaging of endogenous NO by fluorescence microscope

To follow NO accumulation in planta, hypocotyls (1 cm) of cucumber explants grown on plates were incubated in the dark for 2 h in the presence of 20 μM DAF-FM DA (4-amino-5-methylamino-2,7-diamino-fluorescein diacetate, sigma) prepared in 50 mM Tris-HCl (pH 7.5). Hypocotyls were extensively washed with distilled water to remove excess of the fluorophore. The hypocotyls under each condition were observed with a fluorescent microscope (Leica 400x, Planapo, German Weizla). Fluorescence intensity was analyzed using ImageJ software and expressed as a percentage of the control. The experiment was repeated three times and ten hypocotyls were observed each time.

Determination of transcript abundance by real-time PCR

Total RNA was isolated from 100 mg (fresh-weight) of excised cucumber hypocotyls (segments 1 cm long from the base of the hypocotyl), ground with mortar and pestle in liquid nitrogen, using plant RNA extraction kit (TaKaRa MiniBEST 9769; TaKaRa Biomedicals, Japan) according to the manufacturer's instructions. Synthesis of cDNA was performed with Prime Script™ RT reagent Kit (TaKaRa Biomedicals, Japan) starting from 500 ng of total RNA according manual's instructions. The real time quantitative RT-PCR was used to analyze the relative expression of NR genes in hypocotyl of cucumber explant through a SYBR Premix Ex Taq II (Tli RNaseH Plus; TaKaRa Biomedicals, Japan). *Actin* gene (accession number: DQ641117) was used as an internal control. Gene-specific primers were designed by Primer3plus as followed: for NR (accession number: JQ692875.1), forward 5' -AAACCCTACATCCTTCACTCTCG - 3' and reverse 5' -GGTCCATTGCCATTTCTTCT - 3', for

actin, forward 5' -CCCATCTATGAGGGTTACGCC-3' and reverse 5' -TGAGAGCATCAGTAAGGTCACGA-3'. The total volume of each reaction was 20 μL, and contained 10 μL SYBR Premix Ex Taq II, 2 μL of 10-fold diluted cDNA and 0.8 μL of 10 μM forward and reserve primers, to a final volume of 20 μL by adding water. Amplification program consisted of one cycle of 95 °C for 60 s, 40 cycles of 95 °C for 5 s, and melting analysis at 60 °C for 20 s, and 95 °C for 15 s, followed by one cycle of 60 °C for 60 s, and 95 °C for 15 s. All qRT-PCR for each gene was performed in three biological replicates, with three technical repeats per experiment. The relative quantification of mRNA levels is based on the method of Livak and Schmittgen [37]. The threshold cycle value (Ct) of *actin* was subtracted from that of the target gene to obtain a ΔCt value. The Ct value of the control sample in experiment was subtracted from the ΔCt value to obtain a ΔΔCt value. The expression level relative to the control for each sample was expressed as 2^{-ΔΔCt}.

Statistical analysis

Each experiment was repeated three times and the data collected were expressed as mean values ± 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 ($P < 0.05$).

Abbreviations

24-EBR: 24-epibrassinolide; BL: epibrassinolide; BR: Brassinolide; BR₂: Brassinazole; c-PTIO: 2-(4-carboxy-2-phenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide; CTK: cytokinin; H₂O₂: hydrogen peroxide; IAA: indole-3-acetic acid; JA: jasmonic acid; L-NAME: N-nitro-L-arginine methyl ester; NO: Nitric oxide; NOS: NO synthase; NR: Nitrate reductase; SA: salicylic acid; SNAP: S-nitroso-N-acetylpenicillamine

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Authors' contributions

YL and JY2 conceived and designed the research. YL and WL conducted the experiments. YL, YW, XJ, JY1 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 generated during the current study are available from the first author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹College of Horticulture, Gansu Agricultural University, Lanzhou 730070, People's Republic of China. ²Department of Horticulture, FoA, University for Development Studies, P. O. Box TL 1882, Tamale, Ghana.

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