Title: Hydrogen Sulfide Treatment Promotes Root Organogenesis in *Ipomoea batatas*, *Salix matsudana* and *Glycine max*

Running title: Hydrogen Sulfide Promotes Root Organogenesis

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Hydrogen Sulfide Treatment Promotes Root Organogenesis in Ipomoea batatas, Salix matsudana and Glycine max

Abstract: In this report, we demonstrate that NaHS, a hydrogen sulfide (H$_2$S) donor promoted the adventitious root formation mediated by auxin and nitric oxide (NO) signal transduction. Application of H$_2$S donor to seedling cuttings of sweetpotato (Ipomoea batatas) could promote the number and the length of adventitious roots in a dose-dependent manner. It was also verified that H$_2$S or HS$^-$ rather than other sulfur-containing components derived from NaHS was attributed to the progressive role in adventitious root formation. Rapid increment of endogenous H$_2$S, IAA and NO were sequentially observed in shoot tips of sweetpotato seedling treated with the exogenous H$_2$S donor. Further investigations showed that the H$_2$S-mediated root formation was alleviated by N-1-naphthylphthalamic acid (NPA), an IAA transport inhibitor, and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), an NO scavenger. Similar phenomena in H$_2$S donor-dependent root organogenesis were observed in both excised willow (Salix matsudana) shoots and soybean (Glycine max) seedlings. These results indicated that the process of H$_2$S-induced adventitious root formation was probably mediated by the IAA and NO signals and H$_2$S might act upstream of IAA and NO signal pathways.

Key words: auxin; hydrogen sulfide; nitric oxide; root organogenesis; sweetpotato (Ipomoea batatas)
Root organogenesis is a significant event in higher plants. Adventitious rooting, a key step in clonal propagation providing efficient fixation on the substrate and good uptake of water and nutrients from the soil, involves in the development of a meristematic tissue after removal of the primary root system. Adventitious root formation is a complex process that is affected by various environmental factors and multiple endogenous factors including different phytohormones and signal molecules. The plant hormone auxins have been shown to promote this process through the dedifferentiation of cells to reestablish the new apical meristem (Doerner 2008). Although a variety of components of auxin transport and signal transduction were identified, the molecular mechanism underlying the initiation of new root meristems is poorly understood (Doerner 2008; Berleth and Sachs 2001).

Nitric oxide (NO) is a diffusible multifunctional second messenger first described in animals, where it plays variable functions ranging from dilation of blood vessels to neurotransmission and defense during immune response. Recent discoveries have extensively shown its presence and functionality in the plant root formation, except for flowering, germination, senescence, maturity, and programmed cell death. For example, it has been proved that NO acts as a signal molecule in the hormonal cascade leading to root formation (Pagnussat et al. 2004) and is involved in the Azospirillum brasilense-induced lateral root formation in the tomato (Creus et al. 2005). Pagnussat et al. demonstrated that NO mediates the auxin response which results in adventitious root formation (Pagnussat et al. 2002, 2003). Furthermore, a transient increase in NO concentration was observed in hypocotyl cuttings of cucumber (Cucumis sativus), showing that NO is required and part of the molecular events involving adventitious root development induced by indole acetic acid (IAA) (Pagnussat et al. 2002, 2003, 2004). Recently, NO was found to increase the activities of calcium dependent protein kinases (CDPK) and phospholipase D and induce cucumber adventitious root formation together with IAA (Lanteri et al. 2006, 2008). More interestingly, many investigations demonstrated that NO acts downstream of carbon monoxide (CO), which is known as another gaseous signal molecule in animals, involving the mechanism of adventitious root formation (Xu et al. 2006; Cao et al. 2007; Xuan et al. 2008).
Hydrogen sulfide (H$_2$S) has been found to be the third “gaseous signal molecule” after NO and CO in animals (Wang 2002). It has been implicated in the induction of smooth muscle relaxation, hippocampal long-term potentiation, brain development, inflammation and neurons antioxidant protection (Hosoki et al. 1997; Wang 2002; Li et al. 2006; Kimura and Kimura 2004). However, there has been little research in H$_2$S biology in plants. In higher plants, H$_2$S is evolved by enzymatic desulfhydration of cysteine and metabolism of sulfite and sulfate (Rennenberg 1983, 1984). H$_2$S emission by higher plants has already been observed in response to an excess sulfur supply to roots or to leaves (Rennenberg 1984, 1989; Sekiya 1982). Short-term exposure of *Brassica oleracea* to atmospheric H$_2$S levels resulted in a decrease in the activity of adenosine 5’-phosphosulphate reductase in the shoot and a maximal 3-fold increase in thiol content occurred after 5 h exposure (Westerman 2001). H$_2$S also serves as a signal molecule to control thiol levels in *Arabidopsis thaliana* (Riemenschneider et al. 2005). More recently, we demonstrated that H$_2$S plays an important role in the antioxidant metabolism counteracting abiotic stresses during wheat seed germination and sweetpotato seedlings growth (Zhang et al. 2008, 2009). Previous researches mentioned above give a hint that the history of H$_2$S in plants is just beginning.

In animals, it has already been demonstrated that gas molecules NO, CO and H$_2$S play important and putative roles in cell signaling. NO and CO have already been identified as signal molecules in plants and found to be involved in root formation (Pagnussat et al. 2002, 2003, 2004; Xu 2006; Cao 2007; Xuan et al. 2008). However, as the third gasotransmitter after NO and CO in animals, whether H$_2$S acts as a second messenger similar to NO and CO involving root organogenesis in plants remains unclear. In this report, we showed some evidence of its new function in the regulation of root formation mediated by NO and IAA signaling in plants.

**Results**

The effect of H$_2$S on the induction of adventitious root formation in sweetpotato seedling cuttings is dose dependent

NaHS, an H$_2$S donor, which was applied to seedling cuttings of sweetpotato (*Ipomoea*...
*batatas*) cultured in water solution, could induce adventitious root organogenesis in a dose dependent manner (Figure 1). It also indicated that treatment with 0.2 mmol/L NaHS had the most optimal effect on the induction of root number (Figure 1A) and root length (Figure 1B) as compared to the control, respectively. However, at high concentrations of NaHS above 1.0 mmol/L no extra positive effect on root number was caused, and even partial inhibition was observed, indicating that higher level of NaHS could be toxic (Figure 1). Furthermore, application of different concentrations of H2S solutions diluted by saturated H2S aqueous solutions was able to replay the role of the H2S donor NaHS in inducing root organogenesis dose-dependently (Figure 1C and D).

**H2S is attributed to the promoting role of NaHS in adventitious root formation**

To verify the role of H2S in the promotion of adventitious root formation induced by NaHS, 0.2 mmol/L Na2S, Na2SO4, Na2SO3 NaHSO4, NaHSO3 and NaAC were used as the controls of Na+ or sulfur-containing components in the same experimental system. These components were not able to induce adventitious root formation in seedling cuttings of sweetpotato as 0.2 mmol/L NaHS did (Figure 2). Application of H2S non-specific scavenger hemoglobin (Hb) did not affect the adventitious root formation, while it could dramatically counteract the progressive effects of NaHS on root formation (Figure 2). Thus, it is concluded that H2S or HS-, rather than other compounds directly or indirectly derived from NaHS, was responsible for the promoting roles of NaHS on adventitious root formation in sweetpotato explants.

**Changes of endogenous H2S, IAA and NO in sweetpotato explants**

Considering that IAA and NO were involved in root organogenesis, endogenous H2S, IAA, and NO in shoot tips of sweetpotato explants treated with NaHS or water (control) were investigated with time course. A transient increase of endogenous H2S was observed in explants either treated with NaHS or water for 1 day, but H2S content in NaHS-treated explants was significantly higher than that of water culture. Thereafter the levels of endogenous H2S in both explants significantly dropped at 1 day, while higher level of
endogenous H$_2$S in NaHS-treated explants was sustained till the end of experiment (8 d) as
compared with that cultured in water control (Figure 3A). Tendency in changes of
endogenous IAA and NO were similar to that of H$_2$S (Figure 3B and C). Transient increases
in endogenous IAA and NO were observed in both treatments, and then the levels of the
two molecules decreased in both treatments, while higher levels of IAA and NO were kept
in explants treated with NaHS than those cultured in water control (CK). Moreover, the
maximum values of endogenous IAA and NO were observed at the point of 2 and 3 days,
respectively (Figure 3B and C), indicating endogenous H$_2$S, IAA and NO sequentially
appeared in response to H$_2$S donor application. Furthermore, explants treated with NO
donor SNP could keep higher level of endogenous H$_2$S in comparison with the water
control (Figure 3D), inferring that a feedback mechanism be operating for the induction of
root formation.

IAA and NO are involved in the H$_2$S-induced adventitious root organogenesis of
sweetpotato seedlings

Because it showed that endogenous IAA and NO are increased in NaHS-treated explants,
we further investigated that root organogenesis in sweetpotato seedlings treated with either
exogenous NO donor SNP or IAA combined with or without their inhibitors, which were
2-(4-carboxyphenyl)-4,4,5,5- tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO)
and N-1-naphthylphthalamic acid (NPA), respectively. Treatments with SNP, IAA and
NaHS resulted in significant increment in adventitious root number (Figure 4A) and root
length (Figure 4B). On the other hand, both NO scavenger cPTIO and IAA transport
inhibitor NPA could significantly inhibit adventitious root formation in comparison with
the water control (Figure 4). Application of NO scavenger cPTIO could dramatically
counteract the progressive effects of the three compounds SNP, IAA and NaHS, while IAA
transport inhibitor NPA could not result in the same responses to alleviate the effects of
both exogenous IAA and NO donor SNP. NPA could only eliminate the positive roles of
NaHS in the induction of root number and root length. Therefore, either cPTIO or NPA
treatment could apparently inhibit the formation of adventitious roots induced by H$_2$S
donor NaHS (Figure 4). Hb, which is a non-specific scavenger not only for H$_2$S but also for NO, played no positive role on root formation, but could significantly eliminate the promotive effects of exogenous H$_2$S and NO donors application. However, it could not alleviate this physiology function of IAA, mainly because it was a biological large molecule and could not easily transfer into the plant (Figure 4). The above results demonstrated that NO and IAA were involved in H$_2$S-mediated adventitious root organogenesis.

**Root organogenesis in cut shoot of willow induced by H$_2$S donor NaHS is in a dose dependent manner and is involved in NO and IAA signal pathways**

In the present study, cut shoots of willow were also selected as the experimental material to investigate adventitious root organogenesis induced by H$_2$S. As shown in Figure 5A and B, the number and the length of adventitious roots increased significantly after 8 days of treatment with appropriate NaHS. Optimally promotive dosage was 0.2 mmol/L, while the concentration was higher than 0.2 mmol/L positive effects disappeared. In Figure 5C, it further confirmed the possibility that NO and auxin were involved in adventitious root organogenesis induced by H$_2$S. Treatment of willow explants with NaHS plus cPTIO or NPA resulted in a significant reduction in adventitious root formation induced by H$_2$S donor NaHS (Figure 5C). The induction of adventitious root formation by exogenous NO donor SNP and IAA was diminished by NO scavenger cPTIO, but was not affected by auxin transport inhibitor NPA. Hemoglobin which can scavenge H$_2$S and NO could counteract the promotive roles of exogenous NaHS and SNP on root formation. Furthermore, when applied alone, cPTIO or NPA significantly inhibited the formation of adventitious root as compared to the control (Figure 5C), indicating that endogenous NO and auxin signals were involved in adventitious root formation.

**Another proof in the similar mechanism of root organogenesis induced by H$_2$S donor NaHS is obtained in soybean seedlings**

Consistent with the results obtained in sweetpotato and willow mentioned above, the
similar mechanism of lateral root organogenesis induced by H$_2$S donor NaHS was observed
in soybean seedlings. In Figure 6A and B, the results showed that the exogenous H$_2$S donor
NaHS could promote the formation and growth of lateral roots and its effect was dose
dependent. Compared with the other plant materials mentioned above, lower concentrations
of NaHS could promote lateral root organogenesis in the soybean seedlings. Treatment of
seedling explants with 0.01-0.04 mmol/L NaHS significantly enhanced the number of roots
and the optimal concentration of NaHS was 0.02 mmol/L (Figure 6C). NO scavenger
cPTIO eliminated the lateral root-promoting effects of exogenous NO donor SNP, IAA, and
H$_2$S donor NaHS, indicating that NO may function downstream in auxin and H$_2$S signal
pathways and be involved in auxin or H$_2$S-induced root formation (Figure 6C). IAA
transport inhibitor NPA could not block the lateral root-promoting effect of exogenous NO
donor SNP or exogenous IAA, but could counteract the effect of H$_2$S donor NaHS.
Furthermore, auxin transport inhibitor NPA or NO scavenger cPTIO application alone
could inhibit lateral root organogenesis in soybean seedlings (Figure 6C), again suggesting
that endogenous NO and IAA participated in lateral root formation.

Discussion

In this report, we demonstrate that either NaHS, an H$_2$S donor or gaseous H$_2$S aqueous
solutions was able to dose-dependently promote the root formation in plants (Figure 1;
Figure 5A, B; Figure 6A, B) in the same way that NO donor SNP and IAA treatment did
(Figure 4; Figure 5C; Figure 6C). NaHS has been widely used for H$_2$S donor in animals
(Hosoki et al. 1997; Kubo et al. 2007). It dissolves in water and then dissociates to Na$^+$ and
HS$^-$ in solution and HS$^-$ associates with H$^+$ and produces H$_2$S. Thus, in this paper NaHS
was chosen as an H$_2$S donor. Other sulfur-containing components, such as S$^2-$, SO$_4^{2-}$, SO$_3^{2-}$,
HSO$_4^-$, HSO$_3^-$, and Na$^+$, which were used in this paper as the controls of the H$_2$S donor
NaHS, were not able to improve the formation of adventitious root as well as NaHS did
(Figure 2). Furthermore, hemoglobin which can scavenge H$_2$S could counteract the
promotive roles of NaHS on root formation (Figure 2; Figure 4; Figure 5C; Figure 6C).
Those results verify that H$_2$S or HS$^-$, rather than other compounds derived from NaHS,
plays the potential role in promoting adventitious root formation in plants. Moreover, a rapid increase in endogenous H$_2$S in sweatpotato explants treated with or without NaHS was observed at early stage of treatment, further implying that H$_2$S is indeed involved in root formation (Figure 3A). H$_2$S at higher levels in plants is toxic and can block or slow down cell division activity in the apical dominance or change the entire hormonal distribution pattern. For example, excess H$_2$S negatively affects plant growth by inhibition of mitochondrial electron transport (De Kok, 2002; Beauchamp et al. 1984). However, investigations of plasma membrane integrity and MDA content (data not shown), which are two toxic index and indicators of lipid peroxidation in cell, tissue, organ, or the whole plant when subjected to damages, in root and shoot tips, indicated that H$_2$S donor NaHS concentrations (as for *Ipomoea batatas* and *Salix matsudana*, ≤1.0 mM; as for *Glycine max*, ≤ 0.1 mM ) used in our experiments caused no toxic effect on root or shoot cells. These evidences confirmed that NaHS promotes root formation in a way independent of “secondary-response”.

Adventitious root formation is a complex process involving the intricate network of signal molecules, one of them auxin. Auxin transport likely plays an important role in regulating the hormone flux between IAA source and sink tissues, thereby influencing root development. Auxin induces dedifferentiation of parenchyma cells and entrance to cell division to form the root meristem (De Klerk et al. 1995). Even though auxin production has been thoroughly studied, it is still not clear to what extent IAA functions in the root formation and how it is regulated. Interestingly, the action of auxin is coordinated with other plant hormones, such as gibberellin, abscisic acid (ABA), cytokinin, ethylene, and other signal molecules. For instance, Pagnussat et al. (2002) primarily discovered that NO donors, sodium nitroprusside (SNP) and S-nitroso, N-acetyl penicillamine (SNAP), promoted the adventitious root formation in cucumber seedlings, a similar response to that obtained with IAA. Thereafter, more and more investigations demonstrated that NO is required for root organogenesis and mediates at the downstream of the auxin response during the rooting process (Pagnussat et al. 2003, 2004; Lanteri et al. 2006, 2008). In this work, NO scavenger cPTIO could completely prevent the adventitious root formation.
induced by exogenous IAA (Figure 4; Figure 5C; Figure 6C), also confirming that NO is an intermediate in the auxin-regulated signaling cascades determining root morphology and physiology. H₂S donor NaHS was able to mimic the stimulatory effects of the auxin IAA and NO donor SNP in inducing root organogenesis. The auxin polar transport inhibitor NPA reduced the stimulatory effect produced by H₂S on adventitious root formation suggesting that auxins are also involved in H₂S-induced effect on rooting. Moreover, treatments with the NO scavenger cPTIO completely blocked H₂S and IAA-induced adventitious root formation (Figure 4; Figure 5C; Figure 6C), indicating that NO is required for the IAA or H₂S-promoted adventitious root formation and involves in the downstream of H₂S and IAA pathways. Interestingly, in consistency with the results that IAA could induce transient increases in endogenous IAA and NO during the adventitious rooting process in cucumbers (Pagnussat et al. 2002), exogenous H₂S donor also induced the transient increase in endogenous H₂S, IAA and NO in sweetpotato explants and they were emergent sequentially after application of exogenous H₂S donor NaHS (Figure 3A, B, C). These results also inferred that endogenous H₂S was involved in the mechanism of adventitious root formation and it may act upstream of IAA and NO signal pathways.

It is interesting that NO serves as a downstream signal of H₂S in root formation in this report (Figure 4; Figure 5C; Figure 6C). However, H₂S is a reducing agent or an antioxidant, while NO is a redox agent in nature. They probably have adverse effects on cysteine residues of proteins or cellular thiol contents. Furthermore, Kubo et al. (2007) demonstrated that NaHS/H₂S could in vitro inhibit nNOS and eNOS most probably through interaction with BH₄ and inhibit iNOS possibly through multiple unknown mechanisms, thereby probably down-regulating the NO level in animal cells. On the other hand, Published data obtained from animal materials have shown that the endogenous production of H₂S from rat aortic tissues is enhanced by NO donor treatment (Zhao et al. 2001). The NO donor also enhances the expression level of cystathionine γ-lyase (CSE), which catalyzes L-cysteine to H₂S, in cultured vascular smooth muscle cells. Hosoki et al. (1997) observed that the vasorelaxant effect of NO donor SNP was enhanced by incubating rat aortic tissues with 30 μmol/L NaHS. On the contrary, pretreating aortic tissues in another
study with 60 μmol/L H₂S inhibited the vasorelaxant effect of SNP. This paradox may be partially explained by the experimental conditions of these studies, including differences in tissue preparations and tension development before the applications of H₂S and NO. In addition, NO is mostly produced in endothelial cell and slightly in smooth muscle cell, while the production tissue source of H₂S is smooth muscle cell but not endothelial cell (Wang 2002). It deduced that in animal the two signal molecules were effectively separated in varies cells and even departmentalized in different sub-cellar locations.

The evidence in animals provided many hints for the similar mechanisms in plants, though the “Cross-Talk” or “Net-Work” among H₂S, NO and other signals or regulators has been little elucidated. The data in this study support the hypothesis that NO is required for the H₂S-promoted adventitious root formation and that the three molecules H₂S, IAA and NO are cellular messengers involving the intimate association with root formation. A serial linkage “H₂S→IAA→NO→rooting” could be distinguished from the event that a same intermediate can be cross-linked by the three signal molecules. However, whether NO or IAA is involved in the feedback mechanism in root formation induced by H₂S and how endogenous H₂S is perceived and transduced into the specific responses need to be further investigated. Apparently, the involvement of H₂S in this signal pathway of root organogenesis opens a more intricate field of research in the plant kingdom.

Materials and methods

Plant material and chemicals

Sweetpotato (Ipomoea batatas L., cv. Xushu 18) were supplied by Xuzhou Sweetpotato Research Center, Chinese Academy of Agricultural Sciences. Similar sweetpotato seedlings were cut and selected for experiments. Seedling leaves were removed and the tips together with three fully expanded leaves from the top were left. Willow (Salix matsudana Koidz. var. tortuosa Vilm) shoot was obtained from the horticulture center in Heifei University of Technology. Similar shoot with the tips and leaves removed was excised and used for experiments. Seeds of the soybean (Glycine max) obtained from Anhui Academy of Agricultural Sciences were surface-sterilized with 0.2% NaClO for 5 min, washed
extensively and germinated in distilled water at 25±1°C for 3 d in the dark followed by 4 d in a 14 h photoperiod (3000 lx). Soybean seedlings with their primary roots removed were used as explants for experiments. NaHS and SNP were used as H₂S and NO donors, respectively. N-1-naphthylphthalamic acid (NPA), hemoglobin (Hb) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) were used as auxin polar transport inhibitor, non-specific H₂S scavenger, and specific NO scavenger, respectively.

**Treatments**

To investigate the optimal effect of H₂S donor NaHS on root organogenesis in sweetpotato seedlings and excised willow shoot, explants were cultured in Hoagland nutrient solutions (5 mmol/L Ca(NO₃)₂·4H₂O, 1 mmol/L KH₂PO₄, 5 mmol/L KNO₃, 2 mmol/L MgSO₄·7H₂O, 46.4 μmol/L H₃BO₃, 9.2 μmol/L MnCl₂·4H₂O, 1 μmol/L ZnSO₄·7H₂O, 0.32 μmol/L CuSO₄·5H₂O, 0.5 μmol/L Na₂MoO₄·2H₂O, pH 6.0) containing 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mmol/L NaHS solutions, respectively. As for soybean seedlings, the NaHS concentration of 0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 mmol/L were used. Thus, the optimal NaHS concentration capable of inducing adventitious root formation in explants was found, at which promoted adventitious root formation most effectively. To verify the hypothesis that NaHS-mediated root formation of the explants could be attributed to H₂S or HS⁻, non-specific H₂S scavenger hemoglobin, Na⁺, and sulfur-containing components, such as Na₂S, Na₂SO₄, Na₂SO₃, NaHSO₄, NaHSO₃, and NaAC were used as the comparisons of the H₂S donor NaHS. Explants of sweatpotato seedlings used for experiments were cultured in water (CK), NaHS at optimal concentration (obtained from above experiments), Na₂S, Na₂SO₄, Na₂SO₃ NaHSO₄, NaHSO₃, or NaAC at the same concentration to NaHS, respectively. After 8 days of treatment, adventitious root number (≥1 mm) was calculated and were presented as mean ±SE (n=30 explants from at least three independent experiments). In all experiments, the explants were maintained at 18±1°C in the dark and at 28±1°C in the light of 190 μmol m⁻² s⁻¹ (12h/12h), and all treatment solutions were renewed per 12 h. In this report, 20 μmol/L of the auxin IAA, 50 μmol/L of the NO donor SNP, 20
μmol/L of NPA, 100 μmol/L of cPTIO, 0.1 g/L Hb were applied to the explants.

**Measurement of endogenous H₂S in shoot tips**

H₂S was determined by formation of methylene blue from dimethyl-\(p\)-phenylenediamine in H₂SO₄ according to the method described by Sekiya et al. (1982). Shoot tips (0.5 g) were ground and extracted in 5 ml phosphate buffer solution (pH 6.8, 50 mmol/L) containing 0.1 mol/L EDTA and 0.2 mol/L ascorbic acid. The homogenate was mixed with 0.5 ml 1 mol/L HCl in a test tube to release H₂S, and H₂S was absorbed in zinc acetate (0.5 ml 1%) trap which is located in the bottom of the test tube. After 30 min of reaction, 0.3 ml 5 mmol/L dimethyl-\(p\)-phenylenediamine dissolved in 3.5 mmol/L H₂SO₄ was added into the trap. Then 0.3 ml 50 mmol/L ferric ammonium sulfate in 100 mmol/L H₂SO₄ was injected in the trap. The amount of H₂S in zinc acetate traps was determined spectrophotometrically at 667 nm after leaving the mixture for 15 min at room temperature. Blanks were prepared by the same procedures with unused zinc acetate solution and known concentration of Na₂S was used to make the calibration curve.

**Determination of NO level**

The NO level was measured according to the method of Murphy and Noack (1994). Shoot tips were incubated with 100 units of catalase and 100 units of superoxide dismutase for 5 min to remove endogenous ROS before addition of oxyhaemoglobin (10 mmol/L). After 3 min incubation, NO was quantified by spectrophotometric measurement of the conversion of oxyhaemoglobin to methaemoglobin.

**Determination of IAA by HPLC**

Extraction and analysis of IAA in shoot tips of explants were followed with Štefančič et al (2007).

**Statistical analysis**
Significances were tested by one-way or two-way ANOVA, and the results are expressed as the mean values ± SD of three independent experiments. Each experiment was repeated at least three times.

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References


**Figure Legends**

**Figure 1.** The effects of the H$_2$S donor (A, B) and diluted H$_2$S saturated solution (C, D) on adventitious root number (A, C) and length (B, D) in *Ipomoea batatas* explants. Seedling cuttings of sweetpotato (*Ipomoea batatas*) were cultured in 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mmol/L NaHS or 0, 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10%, 50%, 100% diluted H$_2$S saturated solution for 8 days, respectively. Treatment solutions were renewed per 12 h. Adventitious root number (≥1 mm) was calculated after 8 d of treatment and were expressed as mean ±SE (n=30 explants from at least three independent experiments).

**Figure 2.** H$_2$S or HS$^-$ but not other compounds derived from NaHS is attributed to the promoting role in adventitious root formation. Explants were cultured in H$_2$O (CK), 0.2 mmol/L NaHS, 0.2 mmol/L Na$_2$S, 0.2 mmol/L Na$_2$SO$_4$, 0.2 mmol/L Na$_2$SO$_3$, 0.2 mmol/L NaHSO$_4$, 0.2 mmol/L NaHSO$_3$, 0.2 mmol/L NaAC, 0.1 g/L Hemoglobin (Hb) and 0.2 mmol/L NaHS plus 0.1 g/L Hb respectively. Treatment solutions were renewed per 12 h. After 8 days of treatment, adventitious root (≥1 mm) was investigated and root number (A) and root length (B) values were expressed as mean ±SE (n=30 explants from at least three independent experiments). Different letters above the bars indicate significant differences among treatments at the $P<0.05$ level according to the LSD test.

**Figure 3.** Changes of endogenous H$_2$S (A, D), IAA (B), and NO (C) in sweetpotato explants treated with H$_2$S donor NaHS (A, B, C) or NO donor SNP (D). Explants were cultured in H$_2$O (CK), 0.2 mmol/L NaHS, and 50 μmol/L SNP for 8 days. Treatment solutions were renewed per 12 h and changes of endogenous H$_2$S, IAA, and NO in sweetpotato seedling tips were determined with time course.

**Figure 4.** IAA and NO are involved in the H$_2$S-induced formation of adventitious root in sweetpotato explants. In this experiment, 0.2 mmol/L of NaHS, 20 μmol/L of IAA, 50 μmol/L of the NO donor SNP, 20 μmol/L of NPA, 100 μmol/L of cPTIO, 0.1 g/L Hb, alone or combined were applied to the explants. Treatment solutions were renewed per 12 h.
After 8 days of treatment, adventitious root (≥1 mm) was investigated and root number (A) and root length (B) values were expressed as mean ±SE (n=30 explants from at least three independent experiments). Different letters above the bars indicate significant differences among treatments at the P<0.05 level according to the LSD test.

Figure 5. Root organogenesis in cut shoot of willow induced by H₂S donor NaHS is in a dose dependent manner and involved in NO and IAA signal pathways. Cut shoot of willow was treated with 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mmol/L NaHS. Treatment solutions were renewed per 12 h. After 8 d of treatment, (A) photograph of adventitious root formation in cut shoot of willow was taken and (B) root numbers (≥1 mm) was calculated and were expressed as mean ±SE (n=30 explants from at least three independent experiments). (C) IAA and NO were involved in the H₂S-induced formation of adventitious root in willow explants. In this experiment, 0.2 mmol/L of NaHS, 20 μmol/L of the IAA, 50 μmol/L of the NO donor SNP, 20 μmol/L of NPA, 100 μmol/L of cPTIO, 0.1 g/L Hb, alone or combined, were applied to the explants. Root number were expressed as mean ±SE (n=30 explants from at least three independent experiments). Different letters above the bars indicate significant differences among treatments at the P<0.05 level according to the LSD test.

Figure 6. Root organogenesis in seedlings of soybean induced by H₂S donor NaHS is in a dose dependent manner and involved in NO and IAA signal pathways. Soybean seedlings were treated with 0.0, 0.01, 0.02, 0.04, 0.06, 0.08, 0.10 mmol/L NaHS. Treatment solutions were renewed per 12 h. After 8 d of treatment, (A) Photograph of root formation in seedlings of soybean was taken (Treatments with 0.0 (CK) and 0.02 mmol/L NaHS (T) were compared in A’). (B) Root numbers (≥1 mm) was calculated and were expressed as mean ±SE. (C) IAA and NO were involved in the H₂S-induced formation of adventitious root. In this experiment, 0.02 mmol/L of NaHS, 20 μmol/L of the IAA, 50 μmol/L of the NO donor SNP, 20 μmol/L of NPA, 100 μmol/L of cPTIO, 0.1 g/L Hb, alone or combined, were applied to the explants. Root number were expressed as mean ±SE. Different letters above the bars indicate significant differences among treatments at the P<0.05 level.
according to the LSD test.
Figure 1

A

Root number explant

0 0.1 0.2 0.4 0.6 0.8 1

NaHS concentration (mM)

B

Root length (mm)

0 0.1 0.2 0.4 0.6 0.8 1

NaHS concentration (mM)

C

Root number explant

0 0.01 0.05 0.1 0.5 1 5 10 50 100

H2S saturated solution (%)
Figure 2

A

Root number
explant$^{-1}$

B

Root length
(m m)

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<th>Root Number</th>
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<tr>
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<td>NaHS+Hb</td>
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</table>
Figure 3

A) Content of H₂S (μmol·g⁻¹ DW) over treatment time (d)

B) Content of IAA (μmol·g⁻¹ DW) over treatment time (d)

C) Content of NO (μmol·g⁻¹ DW) over treatment time (d)
Content of H₂S (μmol g⁻¹ DW)

- CK
- SNP

D

Treatment time (d)
Figure 5

A

B

C

Root number explant

NaHS concentration (mM)

Root number explant
Figure 6

A

B

C

Root number

explant \(^{-1}\)

NaHS concentration (mM)

Root number

explant \(^{-1}\)

NaHS concentration (mM)