

Sodium nitroprusside promotes multiplication and regeneration of *Malus hupehensis* in vitro plantlets

Xiaojiao Han · Hongqiang Yang · Kaixuan Duan ·
Xinrong Zhang · Haizhou Zhao · Shuzhen You ·
Qianqian Jiang

Received: 29 November 2007 / Accepted: 21 September 2008 / Published online: 14 October 2008
© Springer Science+Business Media B.V. 2008

Abstract The effects of sodium nitroprusside (SNP) on the multiplication, regeneration and rooting of *Malus hupehensis* Rehd. var. *pinyiensis* Jiang in tissue culture have been investigated. The results showed that the multiplication of plantlets was promoted significantly by applying 20 μM SNP to the Murashige and Skoog (MS) medium containing 2.0 μM 6-benzylaminopurine (BA) and 1.0 μM zeatin (ZT). Multiplication of plantlets from the 1st subculture was more sensitive to SNP than that from the 4th or 7th subculture. The differentiation and regeneration of adventitious shoots from leaves or cotyledons increased significantly when 20–30 μM SNP was supplied to the medium MS containing 25 μM BA, 2.5 μM α -naphthaleneacetic acid (NAA) and 2.5 μM ZT. Adventitious shoots regeneration frequency from cotyledons was higher than that from leaves at the presence of SNP. The rooting of plantlets was promoted by SNP significantly and the best result for rooting was achieved in the half-strength MS medium containing 75 μM SNP. In addition, adventitious roots without callus distributed at the base of shoots when SNP was supplied.

Keywords *Malus hupehensis* · Multiplication · Sodium nitroprusside · Nitric oxide · Regeneration

Abbreviations

BA	6-Benzylaminopurine
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	α -Naphthaleneacetic acid
NO	Nitric oxide
SNP	Sodium nitroprusside
ZT	Zeatin

Introduction

Malus hupehensis Rehd. var. *pinyiensis* Jiang is a woody plant originated from China, a variety of *M. hupehensis* Rehd. (tea crabapple). It is highly capable of resisting water-logging, shade, cold, and disease (Li 2001). Thus, it is often used as a rootstock for propagating apples. As PYTC is apogamic, plants are uniform, and little variation is observed among progeny. Moreover, it is amenable for genetic transformation, and therefore ideally suited for genetic studies (Yang and Jie 1997). It is well known that successful transformation of woody fruit trees relies on a good regeneration system (Gómez-Lim and Litz 2004; Petri and Burgos 2005), but an efficient regeneration system has not been previously reported for *M. hupehensis*.

Adventitious shoot and root regeneration is a key step of the establishment of regeneration system of plant. Recently, it has been found that nitric oxide as a novel messenger molecule can regulate plant growth and development (Neill et al. 2003). Pagnussat et al. (2002) and Huang and She (2003) found that exogenous NO could induce adventitious root formation in cucumber and mung bean hypocotyl cuttings. Besides, Correa-Aragunde et al. (2004) also demonstrated that NO played key role in determining lateral root development in tomato. Tun et al. (2001) reported that

X. Han · H. Yang (✉) · K. Duan · X. Zhang · H. Zhao ·
S. You · Q. Jiang
College of Horticulture Science and Engineering, Shandong
Agricultural University, Taian, Shandong 271018, China
e-mail: hqyang@sdau.edu.cn

H. Yang
State Key Laboratory of Crop Biology, Taian, Shandong
271018, China

cytokinin could induce release of NO in plant cell cultures, such as *Arabidopsis*, tobacco and parsley. In addition, NO could also promote the growth of leaves in peas (Leshem 1996) and induce the elongation of root tip in maize (Gouvea et al. 1997). All these indicated that NO might play a role in cell division and therefore participate in adventitious shoot regeneration and multiplication.

In view of the particularity of *M. hupehensis* and the importance of NO, this study was to elucidate effects of NO on proliferation and regeneration of *M. hupehensis* and to lay the foundation for the future genetic transformation of *M. hupehensis*.

Materials and methods

Plant materials

The plantlets and cotyledons of *M. hupehensis* seedlings were used as source material. The plantlets were selected after subculture for approximately 25 days. The first 1–3 expanded leaves were separated from subculture plantlets; the leaf edges were excised, and then cultured in the regeneration medium. All cotyledons were surface-sterilized in 75% ethanol for 30 s, and rinsed three times with sterile distilled water. Subsequently they were immersed in a 0.1% HgCl₂ solution containing 2–3 drops of Tween-20 for 6 min and rinsed five times in sterile distilled water. The sterilized cotyledons were placed in the regeneration medium. The subculture medium consisted of Murashige and Skoog (MS; Murashige and Skoog 1962) supplemented with 2.0 μM 6-benzylaminopurine (BA), 1.0 μM zeatin (ZT) and 1.0 μM α-naphthaleneacetic acid (NAA). Subculture was performed every 30 days on the same medium.

Experimental design

Culture media and culture conditions

MS medium was used as basal medium in all experiments. Every medium was supplemented with 3% (w/v) sucrose and solidified with 0.65% (w/v) agar (Solarbio, Beijing, China). All media were adjusted to pH 5.8–6.2 with 1 N KOH or 1 N HCl before autoclaving at 120°C, 1 atm for 20 min. Sodium nitroprusside (SNP) was as the NO donor. All growth regulators except sodium nitroprusside (SNP) were added before autoclaving. SNP was added after autoclaving by filtering. All cultures except control were kept in dark at 23–25°C in the first 6–7 days and later under a 16/8 h (day/night) photoperiod with a light intensity of 50 μM m⁻² s⁻¹ provided by cool white fluorescent tubes.

Shoot multiplication

The basal multiplication medium was MS medium containing 2.0 μM BA and 1.0 μM ZT. Plantlets from the 1st, 4th or 7th subculture about 2–3 cm high were separated, and then were transferred to the multiplication medium with various SNP concentrations (0, 5, 20, 30, 40 or 60 μM). Mean number of shoots per plantlet were recorded after 25 days.

Adventitious shoots regeneration from leaves or cotyledons

Separated leaves and the sterilized cotyledons were cultured in the regeneration medium containing 25 μM BA, 2.5 μM ZT and 2.5 μM NAA supplemented with different SNP concentrations (0, 10, 20, 30, 40 or 50 μM) respectively. Initial cultures were incubated for 15 days in the dark and subsequent culture in the light. The average number of shoots per leaf or cotyledon and the adventitious shoots regeneration frequency were calculated after 50 and 40 days of treatment, respectively.

Adventitious root formation

The isolated shoots with 2–4 cm long collected from subculture medium were harvested and transferred to the half-strength MS medium supplemented with various SNP concentrations (0, 25, 50, 75 or 100 μM), 2.5 μM IBA or 1.5 μM NAA. Rooting percentage, number of roots and root morphology were determined after 40 days of treatment.

Data collection and statistical analysis

The experiment was repeated three times, with each experiment consisting of 30 explants per treatment. The average number of shoots was presented as the mean shoots regenerated from leaves or cotyledons. Regeneration percentage was expressed as the average percentage of leaves or cotyledons that developed shoots divided by the number of total leaves or cotyledons. ANOVA for statistical analysis and shortest significant ranges (SSR) for multiple comparisons were computed using the SAS software program (SAS Institute Inc. 1999). Standard errors of the difference between treatments were presented.

Results

Effects of SNP on shoot multiplication

The effects of different SNP concentrations on shoot multiplication were detected (Fig. 1). Figure 1 showed that

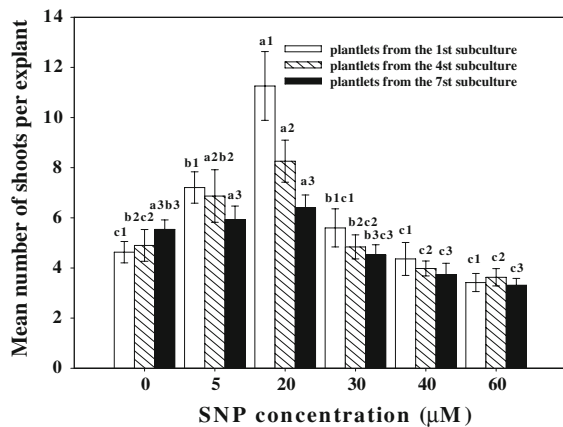


Fig. 1 Effects of various concentrations of SNP on shoots multiplication of *M. hupehensis* var. *pinyiensis* from different subcultures. The other symbols in this figure and that in other figures are similar meaning. Means showing the same letter with number are not significantly different ($P > 0.05$). Vertical bars: standard error

SNP stimulated shoot multiplication in a dose-dependent manner. In the multiplication medium without SNP, the multiplication of plantlets was slower. Mean number of shoots per plantlet increased with the rising of SNP concentrations from 0 to 20 μM . The application of 20 μM SNP significantly induced the increase of multiple shoots compared with the multiplication medium without SNP ($P \leq 0.05$). However, multiplication of plantlets declined when the SNP concentrations were higher than 30 μM .

Furthermore, the role of SNP on shoot multiplication of the plantlets from different subcultures was also recorded in our experiments. Multiplication of plantlets from the 1st subculture was more sensitive to SNP than that from the 4th or 7th subculture. The number of shoots per plantlet from the 1st subculture approached 11.26 at 20 μM SNP, where the number of shoots was markedly higher compared with other treatments ($P \leq 0.05$). There were no significant effects on shoot multiplication of plantlets from the 7th subculture when the SNP concentrations were lower than 20 μM . But higher SNP concentrations (beyond 40 μM) suppressed the shoot multiplication and reduced number of shoots per plantlet. These results showed that multiplication of plantlets from the 1st subculture was more sensitive to SNP than that from the 4th or 7th subculture and high concentration of SNP was adverse to shoot multiplication.

Effects of SNP on adventitious shoots regeneration from leaves

MS media containing different SNP concentrations were tested for adventitious shoots regeneration from leaves (Fig. 2). The SNP from 10 μM to 40 μM induced adventitious shoots regeneration and good development of shoots, especially in the medium containing 30 μM SNP where

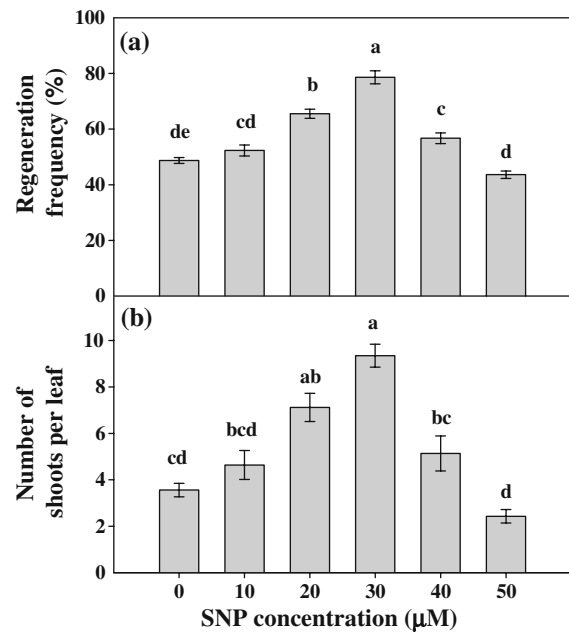


Fig. 2 Effects of various concentrations of SNP on adventitious shoots regeneration from leaves of *M. hupehensis* var. *pinyiensis*. **a** Percent shoot formation. **b** Number of shoots forming on each shoot-producing leaf. Means showing the same letter are not significantly different ($P > 0.05$). Vertical bars: standard error

regeneration frequency and average number of shoots reached 78.6% and 9.35, respectively (Fig. 5a). Therefore, the higher concentration SNP inhibited the regeneration of adventitious shoots, and reduced regeneration frequency and number of adventitious shoots. Leaves became brown and necrotized in the medium with 50 μM SNP (Fig. 5b). These results suggested that SNP also induced adventitious shoots regeneration from leaves in a dose-dependent manner.

Effects of SNP on adventitious shoots regeneration from cotyledons

The effects of SNP on the adventitious shoots regeneration from cotyledons were also detected (Figs. 3, 5c). Figures showed that all media with various SNP concentrations could promote adventitious shoots regeneration, but their effects were different. The number of shoots and regeneration frequency were higher on the MS supplemented with SNP than those in the medium without SNP. Cultured in the medium containing 20 μM SNP, cotyledons produced the highest regeneration frequency (85.5%) and the most shoots. About 20 μM SNP obviously induced adventitious shoots regeneration from cotyledons.

Effects of SNP on adventitious root formation

No adventitious roots formed in the medium without auxins and SNP. However, addition of SNP-induced adventitious

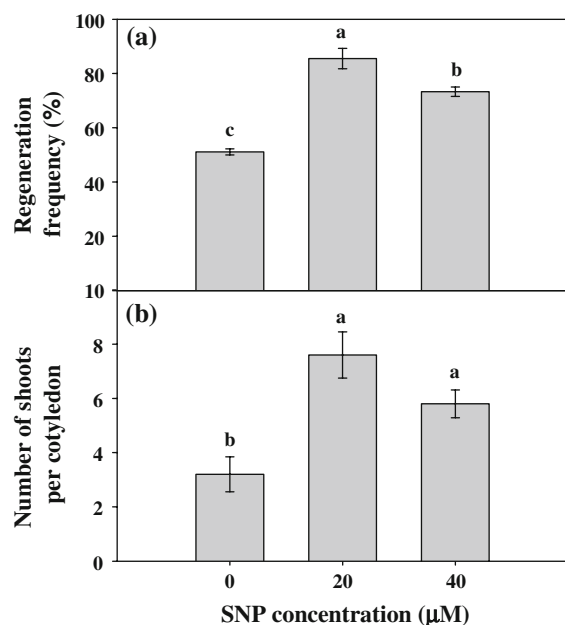


Fig. 3 Effects of different concentrations of SNP on adventitious shoots regeneration from cotyledon of *M. hupehensis* var. *pinyiensis*. **a** Percent shoot formation. **b** Number of shoots forming on each shoot-producing cotyledon. Means showing the same letter are not significantly different ($P > 0.05$). Vertical bars: standard error

root formation significantly. Rooting percentage, number of adventitious roots, number of lateral roots and number of total roots (sum of adventitious roots and lateral roots) gradually increased with the increasing of SNP concentrations from 0 to 75 μM (Fig. 4). Rooting percentage and number of adventitious roots reached 86.2% and 2.5, respectively, in the medium with 75 μM SNP. When SNP concentrations were over 75 μM, the numbers of adventitious roots decreased. The number of lateral roots was highest in the medium containing 100 μM SNP. These results showed that adventitious root formation was more sensitive to the concentration of SNP than the lateral root formation.

About 75 μM SNP was significantly different from 2.5 μM IBA or 1.5 μM NAA for rooting percentage. NAA produced the greatest number of adventitious roots, but no lateral root was found in the adventitious roots. Plantlets cultured in the medium containing 100 μM SNP produced the highest number of lateral roots and the highest number of total roots. This indicated that 100 μM SNP in the half-strength MS medium was more suitable for lateral root formation than 2.5 μM IBA or 1.5 μM NAA.

Effects of SNP on root morphology

Effects of SNP on root morphology were investigated after 40 days of culture (Fig. 5 and Table 1). The MS medium

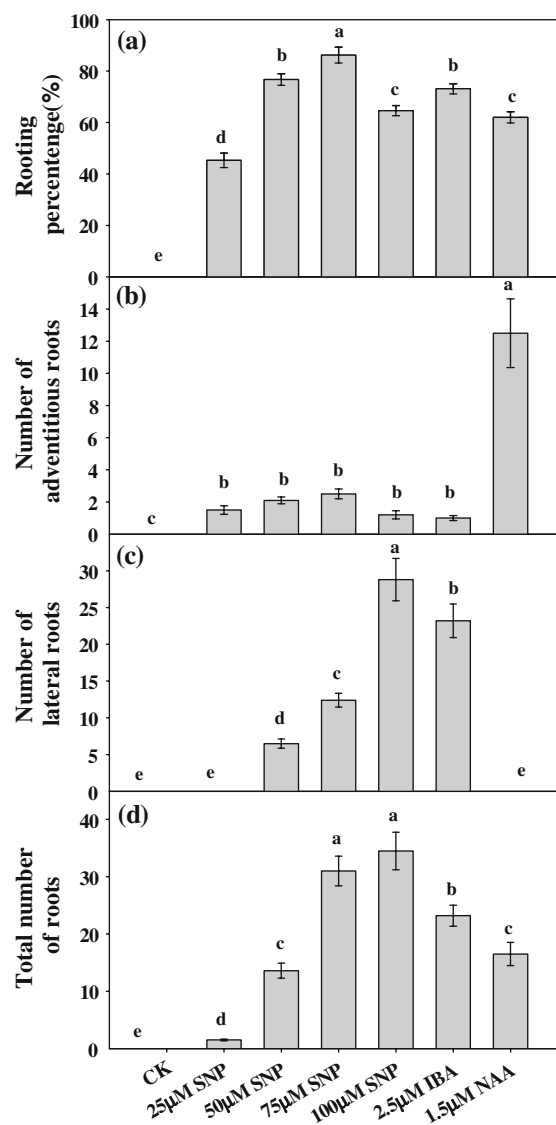


Fig. 4 Effects of SNP on adventitious roots formation of *M. hupehensis* var. *pinyiensis*. **a** Percent root formation. **b** Mean number of adventitious roots per shoot. **c** Mean number of lateral roots per adventitious root. **d** Mean number of total roots per shoot (sum of number of adventitious roots and all lateral roots). Means showing the same letter are not significantly different ($P > 0.05$). Vertical bars: standard error

containing SNP for rooting produced even distribution of lateral roots and longer roots, and the roots formed directly from the shoot base (Fig. 5d). When IBA was added into the medium, fewer adventitious roots and more lateral roots from the adventitious roots were formed, and lateral roots distributed at middle and upper part of the adventitious root (Fig. 5e). Furthermore, NAA resulted in more adventitious roots and no lateral roots in the adventitious roots. The heavy callus at the base of shoots and rooting from the callus were found, and the roots were thick and short in the medium containing NAA (Fig. 5f and Table 1).

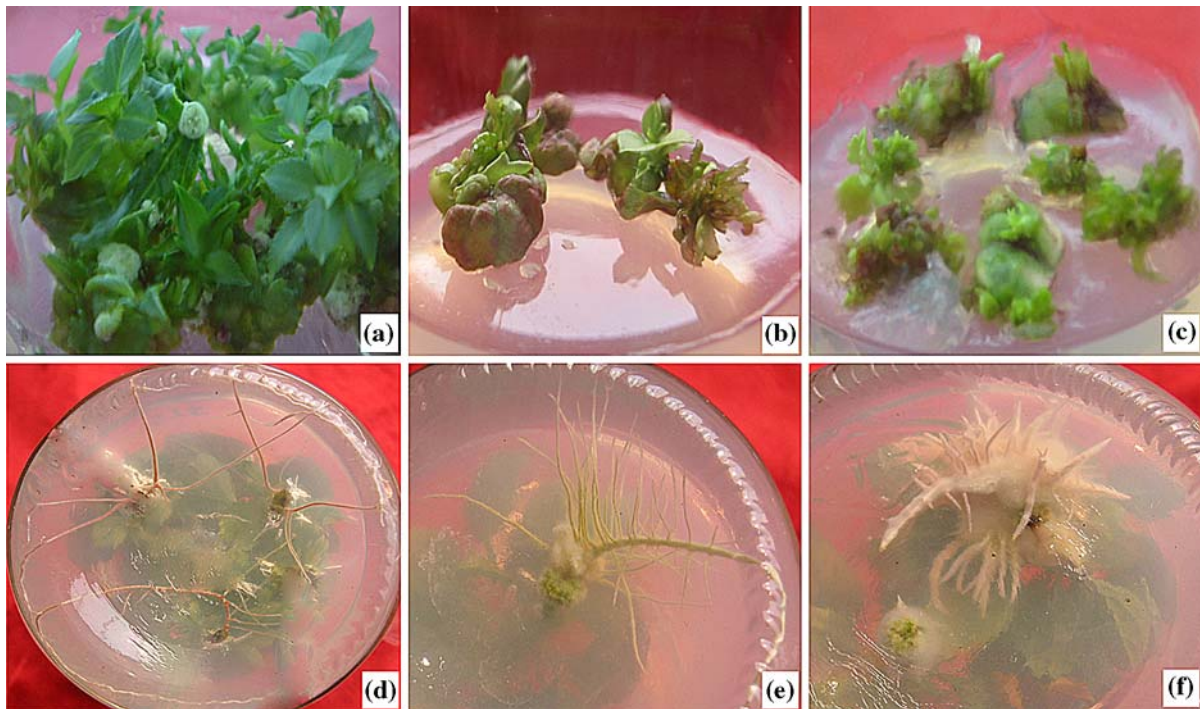


Fig. 5 Adventitious shoots regeneration from leaves and cotyledons, and adventitious roots formation in *M. hupehensis* var. *pinyiensis*. **a** Adventitious shoots regeneration from leaves on the regeneration medium with 30 μM SNP. **b** Adventitious shoots regeneration from leaves on the regeneration medium with 50 μM SNP. **c** Adventitious

shoots regeneration from cotyledons on the regeneration medium with 20 μM SNP. **d** Rooted plantlets on $\frac{1}{2}\text{MS} + 75 \mu\text{M}$ SNP. **e** Rooted plantlet on $\frac{1}{2}\text{MS} + 2.5 \mu\text{M}$ IBA. **f** Rooted plantlet on $\frac{1}{2}\text{MS} + 1.5 \mu\text{M}$ NAA

Table 1 Effects of SNP on root morphology of *M. hupehensis* var. *pinyiensis*

Treatment (μM)	Root color	Lateral roots distribution	Root morphology	Callus at the base of shoots
SNP0 ($\frac{1}{2}\text{MS}$ only)	None	None	None	None
SNP50	Pink	Even	Long and thin	None
SNP75	Pink	Even	Long and thin	None
SNP100	White	Even	Long and thin	None
IBA2.5	Kelly	Middle and upper	From thick to thin	Little
NAA1.5	White	None	Thick and short	Heavy

Comparably, SNP-induced roots presented more similar to the natural morphology than IBA- and NAA-induced roots.

Discussion

A series of physiological changes of plantlets are happening in long-term subculture process. Different stage of subculture has different sensitivity to regulator. Multiplication of plantlets from the 1st subculture was more sensitive to SNP than that from the 4th or 7th subculture (Fig. 1). Carimi et al. (2005) provided the evidence that NO was produced in suspension cell cultures treated with BA in a dose-dependent manner. Figure 1 shows that shoot

multiplication regulated by SNP in a dose-dependent manner too.

The influence of nitric oxide was closely related to plant hormones on the plant growth. Tun et al. (2001) demonstrated rapid increase of NO release in plant cell cultures induced by cytokinin and implied that NO might act in cytokinin signal transduction. Cytokinins regulate a number of important processes including adventitious shoots differentiation and regeneration of plant in vitro. This report demonstrated that SNP obviously induced adventitious shoots differentiation and regeneration from leaves and cotyledons in vitro. The role of NO on adventitious shoots regeneration is poorly understood in previous studies. The mechanism underlying adventitious shoots differentiation

induced by exogenous nitric oxide may be similar. Because exogenous cytokinins led to a rapid stimulation of NO release in cell cultures which has the properties necessary for a potential role in cytokinin signal transduction (Tun et al. 2001). Therefore, NO can be considered as an ‘intermediary’ of adventitious shoots differentiation and it is more efficient and effective to induce adventitious shoots regeneration.

Adventitious rooting involves the development of a meristematic tissue after removal of the primary root system. More recently, it was demonstrated that NO was involved in the auxin response during adventitious root formation in cucumber (Pagnussat et al. 2002) and a subsequent report showed that an NO-mediated cGMP-dependent pathway was operating in that process (Pagnussat et al. 2003). This report showed that the NO donor of SNP could regulate the formation of adventitious root in woody plant (Fig. 4). Auxin induced dedifferentiation of parenchyma cells and entrance to cell division to form the root meristem (De Klerk et al. 1995; Fujita and Syôno 1996). Auxin and NO have also been suggested to share common steps in signal transduction pathways leading to root elongation (Gouvêa et al. 1997). These indicate that NO may interact with auxins linking the regulation of cell division to differentiation during the “de-differentiation” and “re-differentiation” of plant cells (Ötvös et al. 2005).

Comparative effects of SNP, IBA and NAA on adventitious root formation were remarkably different. SNP promoted rooting resulted in even distribution of lateral roots, no callus at the base of shoots and rooting directly from the shoot base. When IBA was added, it resulted in more lateral roots and middle and upper distribution of lateral roots. The MS medium containing NAA for rooting, it produced heavy callus at the base of shoots and rooting from the callus (Fig. 5d–f). Pagnussat et al. (2002) found that NO- and IAA-induced roots presented similar anatomic structure. These indicate that the effect of NO on adventitious roots is similar to indole auxin (such as IAA and IBA), and is significantly different from naphthalene auxin (such as NAA).

Acknowledgment This study was supported by projects 30571285 and 30671452 of the National Natural Science Foundation of China.

References

- Carimi F, Zottini M, Costa A, Cattelan I, Michele RD, Terzi M et al (2005) NO signalling in cytokinin-induced programmed cell death. *Plant Cell Environ* 28:1171–1178. doi:10.1111/j.1365-3040.2005.01355.x
- Correa-Aragunde N, Graziano M, Lamattina L (2004) Nitric oxide plays a central role in determining lateral root development in tomato. *Planta* 218:900–905. doi:10.1007/s00425-003-1172-7
- De Klerk G-J, Keppel M, Ter Brugge J, Meekes H (1995) Timing of the phases in adventitious root formation in apple microcuttings. *J Exp Bot* 46:965–972. doi:10.1093/jxb/46.8.965
- Fujita H, Syôno K (1996) Genetic analysis of the effects of polar auxin transport inhibitors on root growth in *Arabidopsis thaliana*. *Plant Cell Physiol* 37:1094–1101
- Gómez-Lim MA, Litz RE (2004) Genetic transformation of perennial tropical fruits. *In vitro Cell Dev Biol Plant* 40:442–449
- Gouvêa CMCP, Souza JF, Magalhas ACN, Martins IS (1997) NO-releasing substances that induce growth elongation in maize root segments. *Plant Growth Reg* 21:183–187
- Huang AX, She XP (2003) Effect of SNP on rooting of hypocotyls cutting from mung bean seedling. *Acta Bot Boreali-Occident Sin* 23(12):2196–2199 (in Chinese)
- Leshem YY (1996) Nitric oxide in biological systems. *Plant Growth Reg* 18:155–169
- Li YN (2001) Researches of germplasm resources of *Malus* Mill. China Agriculture Press, Beijing, pp 234–237 (in Chinese)
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497
- Neill SJ, Desikan R, Hancock JT (2003) Nitric oxide signaling in plants. *New Phytol* 159:11–35
- Ötvös K, Pasternak TP, Miskolczi P, Domoki M, Dorjgotov D, Szűcs A, Bottka S, Dudits D, Fehér A (2005) Nitric oxide is required for, and promotes auxin-mediated activation of, cell division and embryogenic cell formation but does not influence cell cycle progression in alfalfa cell cultures. *Plant J* 43:849–860
- Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. *Plant Physiol* 129:954–956
- Pagnussat GC, Lanteri ML, Lamattina L (2003) Nitric oxide and cyclic GMP are messengers in the IAA-induced adventitious rooting process. *Plant Physiol* 132:1241–1248
- Petri C, Burgos L (2005) Transformation of fruit trees. useful breeding tool or continued future prospect? *Transgen Res* 14: 15–26
- SAS Institute Inc (1999) SAS/STAT user’s guide, version 7-1. SAS Institute Inc, Cary, North Carolina
- Tun NN, Holk A, Scherer FE (2001) Rapid increase of NO release in plant cell cultures induced by cytokinin. *FEBS Lett* 509:174–176
- Yang HQ, Jie YL (1997) Studies of individual difference in seedlings of apple rootstock. *J Shandong Agri Univ* 28:487–491 (in Chinese)