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Mori, Yasuhiro
Fukuoka Prefecture Forest Research and Extension Center | Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University

Miyahara, Fumihiko
Fukuoka Prefecture Forest Research and Extension Center

Tsutsumi, Yuji
Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University

Kondo, Ryuichiro
Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University

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Effects of combinational treatment with ethephon and indole-3-butyric acid on adventitious rooting of *Pinus thunbergii* cuttings

Yasuhiro Mori[1,2], Fumihiko Miyahara[1], Yuji Tsutsumi[2*] and Ryuichiro Kondo[2]

[1] Fukuoka Prefecture Forest Research and Extension Center, 1438-2, Toyoda Yamamoto-machi, Kurume, Fukuoka 839-0827, Japan

[2] Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, 812-8581, Japan

*Corresponding author. Ph: +81-92-642-4282. Fax: +81-92-642-4282. E-mail: y-tsutsu@agr.kyushu-u.ac.jp

Abstract

Adventitious rooting is essential for cutting propagation of pine wilt-resistant *Pinus thunbergii*. To examine a variety of adventitious rooting potentials among donor plants, cuttings were taken from 31 seedlings within a half-sib family. Rooting abilities of cuttings from each seedling ranged from 0 to 100%. When 11 ortets and 11 ramets (clonally propagated from each ortet) were used as donor plants, there was a positive correlation between rooting abilities of cuttings from ortets and ramets, suggesting that adventitious rooting is dependent on genetic factors in the donor plants. To promote adventitious rooting of cuttings by hormonal treatment, we examined the effect of soaking time in Oxyberon (19.7 mM indole-3-butyric acid (IBA) solution) on rooting. Ten minutes was the best soaking time for rooted cuttings to produce more adventitious roots without impairing normal growth. When cuttings were soaked in Ethrel diluent (69.2 μM ethephon) for 24 h before soaking in Oxyberon for 10 min, a significantly higher rooting ability was observed than those soaked in Oxyberon alone. Ethrel on its own barely affected rooting ability. The positive effect of the combinational treatment was confirmed in a similar experiment using authentic ethephon and IBA instead of Ethrel and Oxyberon. When cuttings were soaked in a mixture of ethephon and silver thiosulfate (STS), an ethylene action inhibitor, before

IBA-soaking, the effect was partially diminished compared with combinational treatment without STS. These findings suggest that ethylene action caused by ethephon treatment promotes IBA-mediated adventitious rooting of *P. thunbergii* cuttings.

Keywords

Adventitious root · Cutting · Ethephon · Ethylene · Indole-3-butyric acid · *Pinus thunbergii*

Introduction

Pine wilt disease, caused by pine wood nematodes (*Bursaphelenchus xylophilus*), is the most serious disease affecting Japanese and East Asian pine forests. In Japan, large amounts of pine wilt-resistant Japanese black pine (*Pinus thunbergii*) plants are required for reforestation of damaged forests. Seed orchards consisting of 16 clones selected in a resistance breeding project (Fujimoto et al. 1989) have been established for producing resistant plants. However, nearly half of these seedlings have been damaged after inoculation with pine wood nematodes (Goto and Miyahara 2000). In recent years, cutting-propagated plants taken from non-damaged seedlings after inoculation retained very high resistance to pine wilt disease (Mori et al. 2006a,b). Therefore, cutting propagation is expected to be an alternative method to conventional seedling propagation for resistant plant production. However, cuttings from pine trees are generally difficult to root (Kozlowski and Pallardy 1997). Using only a few easy-to-root genotypes as donor plants for cost-effective propagation might reduce genetic diversity in propagated plant populations. Promoting adventitious rooting of *P. thunbergii* cuttings, especially from difficult-to-root genotypes, is therefore desirable.

Adventitious rooting during cutting propagation is affected by multiple factors. These include physiological state (Koukourikou-Petridou 1998), age of donor plants

(Browne et al. 1997), phytohormones (De Klerk 2002), season (Swamy et al. 2002), and genetic characteristics (Greenwood and Weir 1994). In particular, the importance of phytohormone treatment is widely recognized. Auxin plays a central role in adventitious rooting (Blakesley 1994; De Klerk et al. 1999). The synthetic auxins indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) are commercially employed for cutting propagation (Hartmann et al. 1990). For example, cut ends of cuttings are quick-dipped for 2–10 s in IBA or NAA solutions at high concentrations (1.0–32.2 mM) (Goh et al. 1995; Browne et al. 1997; Ford et al. 2002; Dougherty et al. 2004; Sasaki et al. 2004; Henrique et al. 2006).

Ethylene and ethephon which releases ethylene by auto-hydrolysis also promote adventitious rooting in some herbaceous plants, e.g. rice (*Oryza sativa*) (Lorbiecke and Sauter 1999) and mung bean (*Phaseolus radiatus*) (Pan et al. 2002). In wetland plants such as *Rumex palustris*, Visser et al. (1996) found that ethylene-stimulated adventitious rooting was promoted by simultaneous application of NAA, whereas it was counteracted by pre-treatment with *N*-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor. Clark et al. (1999) reported that the ethylene-insensitive *Never ripe* (NR) mutant of tomato (*Lycopersicon esculentum*) showed fewer adventitious roots aboveground or from stem cuttings than wild type; in addition, there was no IBA-dose dependent increase in adventitious roots of NR cuttings. These reports indicate that ethylene also has an important role in adventitious rooting, and that it acts in concert with auxin.

In this study, to propagate pine wilt-resistant *P. thunbergii* plants by cutting, we examined the effects of ethephon pre-treatment on adventitious rooting of auxin-treated cuttings.

Materials and methods

Chemicals

Oxyberon^{TM} was obtained from Bayer CropScience Japan. Ethrel^{TM} was obtained from Nissan Chemical Industries Ltd. Ethephon, IBA, indole-3-acetic acid (IAA), anthranilic acid, silver nitrate and sodium thiosulfate were obtained from Wako Pure Chemical Industries Ltd.

Cutting preparation

Current shoots were sampled from *P. thunbergii* donor plants, before visible bud burst from the end of February to the middle of March. Shoots were soaked in running tap water for 1–2 days at room temperature. Cuttings were prepared by severing 5 cm below the apical buds. Lateral buds and basal needles were removed, and cut ends of cuttings were re-cut at an acute angle just before treatment.

Rooting environment

Cuttings were planted into plastic containers (36 × 51 × 10.5 cm) containing a 3:1 (v/v) mixture of Kanumatsuchi, a popular Japanese rooting media, and vermiculite to a depth of 4.5 cm. Cuttings were cultivated in a greenhouse equipped with an automatic mist watering system at ambient temperature.

Experiment 1: Variation of rooting abilities among donor plants

Thirty-one 8-year-old seedlings within a half-sib family were used as donor plants. Ten cuttings were sampled from each seedling at the end of February 2003. Re-cut faces of cuttings were treated with a talc powder of Oxyberon containing 1.0% IBA before planting.

Experiment 2: Ortet-ramet correlation of rooting abilities

Eleven 10-year-old seedlings (ortets) and eleven 6-year-old plants (ramets) clonally propagated from the ortets were used as donor plants. Ortets and ramets were cultured in separate fields. Eight to ten cuttings per donor plant were sampled at the end of February 2005. The basal 3 cm of cuttings was dipped in Oxyberon (19.7 mM IBA solution) for 5 s before planting.

Experiment 3: Effects of soaking time in Oxyberon on rooting

Six 7-year-old seedlings were used as donor plants. Thirty cuttings were sampled from each seedling and divided into three groups at the end of February 2005. The basal 3 cm of cuttings was soaked in Oxyberon liquid for three different periods of time (5 s, 10 min and 1 h) before planting.

Experiment 4: Effects of soaking in Ethrel on rooting

Ten 8 to 11-year-old seedlings were used as donor plants. Thirty cuttings were sampled from each seedling and divided into three groups at the end of February 2006. The basal 3 cm of cuttings was soaked in the following solutions before planting: (A) distilled water for 24 h, ‘Water’ ; (B) 10,000-fold dilution of Ethrel (69.2 μM ethephon) for 24 h, ‘ETH’ ; (C) Oxyberon for 10 min, ‘IBA’.

Experiment 5: Effects of combinational treatment with Ethrel and Oxyberon on rooting

Ten 11-year-old difficult-to-root seedlings (rooting abilities ranging from 0–30% in Experiment 1) and three 11-year-old easy-to-root seedlings (rooting abilities ranging from 70–100% in Experiment 1) were used as donor plants. Thirty cuttings from each seedling were divided into three groups at the end of March 2006. The basal 3 cm of cuttings was soaked in the following solutions before planting: (A) 10,000-fold dilution of Ethrel for 24 h before Oxyberon for 10 min, 'ETH→IBA' ; (B) Oxyberon for 10 min before 10,000-fold dilution of Ethrel for 24 h, 'IBA→ETH' ; (C) Oxyberon for 10 min, 'IBA alone'.

Experiment 6: Effects of combinational treatment with authentic ethephon and IBA on rooting

Ten difficult-to-root seedlings (the same individuals used in Experiment 5) were used as donor plants. Thirty cuttings from each seedling were divided into three groups in the middle of March 2007. The basal 3 cm of cuttings was soaked in the following solutions before planting: (A) 69.2 μM ethephon in distilled water for 24 h before 19.7 mM IBA in 50% ethanol for 10 min, 'ETH→IBA' ; (B) 19.7 mM IBA for 10 min before 69.2 μM ethephon for 24 h, 'IBA→ETH' ; (C) 19.7 mM IBA for 10 min, 'IBA alone'.

Experiment 7: Effects of silver thiosulfate, indole-3-acetic acid and anthranilic acid on rooting of combinational-treated cuttings

Thirteen 8 to 9-year-old seedlings were used as donor plants. Sixty cuttings from each seedling were divided into six groups in the middle of March 2007. The basal 3 cm of cuttings was soaked in 19.7 mM IBA solution for 10 min after soaking in the following solutions before planting: (A) 69.2 μM ethephon for 24 h, 'ETH→IBA' ; (B) a mixture of 69.2 μM ethephon and 100 μM STS (silver thiosulfate) for 24 h, 'ETH+STS→IBA' ; (C) a complex mixture of 69.2 μM ethephon, 100 μM STS and 10 μM IAA for 24 h, 'ETH+STS+IAA→IBA' ; (D) a complex mixture of 69.2 μM ethephon, 100 μM STS and 10 μM anthranilic acid for 24 h, 'ETH+STS+ANT→IBA' ; (E) 10 μM IAA for 24 h, 'IAA→IBA' ; (F) non-treatment, 'IBA alone'. STS was prepared just before the treatment by mixing 0.1 M silver nitrate and 0.1 M sodium thiosulfate in a ratio of 1:8 (Ichimura and Goto 2002).

Rooting assessments

In all experiments, the rooting ability (percentage of rooted cuttings in planted cuttings) of cuttings per donor plant was assessed 4 months after planting. The means of the rooting abilities were compared among treatments. In Experiment 2, the correlation efficient between rooting abilities of cutting from ortets and ramets was calculated. Percent data were transformed by arcsine. In Experiment 3, adventitious roots per rooted cutting were counted, and the means were compared among treatments.

Results

In Experiment 1, rooting abilities showed a great variation among donor plants within a half-sib family (Fig. 1). Only four out of 31 donor plants exceeded 70%. These plants also showed high percentages (61.8–92.7%) in our previous experiments (Mori et al. 2004). However, three donor plants produced no adventitious roots from their cuttings. These plants also showed low rooting abilities (4.0–30.0%) in our previous study (Mori et al. 2004). This result revealed that rooting abilities of cuttings are dependent on donor-plant. In Experiment 2, there was a positive correlation between rooting abilities of cuttings from ortets and ramets ($r = 0.83$) (Fig. 2).

In Experiment 3, cuttings soaked for 10 min in Oxyberon had the maximum value of rooting ability (78.3%), but there was no significant difference among soaking times ($P > 0.22$) (Fig. 3a). However, there was a significant time-dependent increase in the number of adventitious roots (*P* < 0.01) (Fig. 3b). The area containing emerging adventitious roots spread from the base to the middle portion of cuttings when soaked in Oxyberon for 10 min and 1 h. Unexpectedly, some of the 1 h-soaked cuttings died or had impaired normal elongation of apical buds after rooting; this was not observed in 5 s- or 10 min-soaked cuttings (data not shown). Thus, the best soaking time in Oxyberon was determined to be 10 min.

In Experiment 4, rooting ability was not greatly influenced by Ethrel treatment (7.0%) compared with control treatment (2.0%) (Fig. 4). A significantly higher value (29.0%) was obtained by Oxyberon treatment compared with the control and Ethrel treatment (*P* < 0.05). In Experiment 5, however, the combined Ethrel-Oxyberon treatment had a synergistic effect on rooting of difficult-to-root cuttings. Rooting ability was significantly increased in ETH→IBA (58.0%) compared with IBA alone (40.0%) (*P* < 0.05) (Fig. 5a). However, there was no significant difference between IBA→ETH (34.0%) and IBA alone. Easy-to-root cuttings showed high rooting abilities (77–93%) under every treatment (Fig. 5b). In Experiment 6, using authentic ethephon and IBA instead of Ethrel and Oxyberon, cuttings also showed a significantly higher rooting ability in ETH→IBA (49.4%) compared with IBA alone (33.5%) (Fig. 6).

We attempted to examine how ethephon promoted adventitious rooting in concert with IBA. Stepanova et al. (2005) reported that ethylene treatment up-regulated the expression of *WEI2/ASA1* and *WEI7/ASB1*, genes that encode the α- and β-subunits of anthranilate synthase (a rate-limiting enzyme of tryptophan biosynthesis) in *Arabidopsis thaliana*. They suggested that endogenous auxin (IAA) accumulated via a tryptophan-dependent pathway in roots of ethylene-treated seedlings. Ota (1980) reported that IAA promoted adventitious rooting of cuttings from *P. thunbergii* seedlings. Based on these reports, we speculated that IAA accumulation caused by ethephon-released ethylene synergistically affected adventitious rooting of *P. thunbergii* cuttings treated with exogenous IBA. In Experiment 7, we tested this speculation by using the ethylene action inhibitor STS (Martinis and Mariani 1999, Wagstaff et al. 2005), IAA, and anthranilic acid in addition to ethephon and IBA. A significantly higher rooting ability (47.3%) was obtained in the cuttings treated with ETH→IBA compared with those treated with IBA alone (32.3%), similar to the results of Experiments 5 and 6 ($P < 0.05$) (Fig. 7). This positive effect on rooting was partially diminished by STS, and was not restored by either IAA or anthranilic acid. There was no significant difference between IAA→IBA and IBA alone, which suggests that IAA itself did not synergistically affect adventitious rooting in concert with IBA.

Discussion

Adventitious rooting is an essential step in the vegetative propagation of commercially important plants such as pine wilt-resistant *P. thunbergii*. Browne et al. (1997) compared rooting capacities of *P. banksiana* cuttings among 15 donor plants from six age classes. In each class, they observed both good (easy-to-root) and poor (difficult-to-root) donor plants. Foster (1990) and Greenwood and Weir (1994) noted a genetic variation in rooting ability of *P. taeda* cuttings. In this study, the rooting abilities of *P. thunbergii* cuttings varied greatly among donor plants within a half-sib family (Fig. 1). We previously reported that this variation was not dependent on cutting year (Mori et al. 2004). In another experiment, there was a significant ortet-ramet correlation in rooting abilities (Fig. 2), even though ortets and ramets had been cultured in different fields. Together with the published reports, these results indicate that adventitious rooting of *P. thunbergii* cuttings is dependent on genetic factors in the donor plants.

Auxin is widely applied in the vegetative propagation of various plants. *Arabidopsis* and *P. taeda* explants have been cultured in vitro on media containing 10–100 μM IBA to induce adventitious rooting (Greenwood et al. 2001; Ludwig-Müller et al. 2005). In contrast, researchers have often treated ex vitro woody cuttings with IBA at very high concentrations. For example, *P. caribaea* cuttings quick-dipped in 9.8, 19.7 or 29.5 mM IBA for 2 s showed a maximum rooting ability at 19.7 mM (Henrique et al. 2006). Likewise, quick-dipping in Oxyberon (19.7 mM IBA) has been used for cutting propagation of *P. thunbergii* (Sasaki et al. 2004). However, it has been poorly investigated whether soaking cuttings for a longer time at such a high auxin concentration is effective for adventitious rooting. In this study, rooting ability was not greatly affected by soaking for 10 min or 1 h in Oxyberon compared with quick-dipping (5 s), although there was a time-dependent increase in number of adventitious roots (Fig. 3b). Because some of the 1 h-soaked cuttings were dead or had impaired normal elongation of apical buds after rooting, the best soaking time was determined to be 10 min in Oxyberon.

Ethylene or ethephon treatment promotes adventitious rooting of some herbaceous plant cuttings (Visser et al. 1996; Lorbiecke and Sauter 1999; Pan et al. 2002). Pan et al. (2002) tested concentrations of ethephon ranging from 1 nM to 1 mM, and found that *Phaseolus radiatus* cuttings produced the most adventitious roots after being soaked for 24 h in 50 μM ethephon. Liu and Reid (1992) reported that more adventitious root primordia were observed in decapitated hypocotyls of sunflower (*Helianthus annuus*) that were pre-treated with 69 μM ethephon in combination with

50 μM IAA. Based on these reports and our preliminary experiments, we applied 10,000-fold dilutions of Ethrel (69.2 μM ethephon) for 24 h. Cuttings soaked in 19.7 mM IBA for 10 min after ethephon treatment showed significantly higher rooting abilities than those treated with IBA alone (Figs. 5, 6), even though they were sampled from difficult-to-root donor plants. In support of these results, the combinational treatment also resulted in a significantly higher rooting ability than sole IBA treatment (Fig. 7). Interestingly, when the cuttings were simultaneously pre-treated with ethephon and STS before IBA treatment, rooting ability was partially reduced, compared with the combinational treatment without STS (Fig. 7). According to Zhao et al. (2002), the ethylene receptor ETR1 contains one ethylene-binding site, mediated by a single copper ion. In the presence of ethylene, the receptors are inactivated, thereby relieving repression of the ethylene response pathway. Replacement of the copper cofactor by STS-derived silver locks the receptor into a conformation that continuously represses ethylene responses. In our experiments, ethephon itself had no effects on adventitious rooting (Fig. 4). Thus, it is possible that the ethylene response promoted positive effects on adventitious rooting by IBA. Stepanova et al. (2005) reported that IAA was accumulated by ethylene treatment, which led us to speculate that IAA synergistically affected adventitious rooting with IBA. Contrary to this speculation, the

reduction of rooting ability by STS was not restored by the addition of IAA or anthranilic acid. In addition, IAA had no synergistic effects on adventitious rooting in concert with IBA (Fig. 7). These results suggest that synergistic effects caused by combinational treatment with ethephon and IBA were not mediated through the accumulation of endogenous IAA.

It is interesting to study the mechanisms of how ethephon promotes adventitious rooting in concert with auxin. Recently, Gutierrez et al. (2009) reported that AUXIN RESPONSE FACTOR6 (ARF6) and ARF8 are positive regulators of adventitious rooting in *Arabidopsis* hypocotyls, whereas ARF17 is a negative regulator. Okushima et al. (2005) demonstrated that lateral root formation in *Arabidopsis* is redundantly regulated by two ARF transcription activators, ARF7 and ARF19. Thus, ARFs play essential roles in auxin-mediated root formation from post-differentiated organs. Ethylene and auxin interact during ARF-mediated developmental processes of plants (Li et al. 2004; Li et al. 2006). Li et al. (2006) showed that ethylene treatment activated transcription of ARF19 in roots of *Arabidopsis* seedlings, which might provide an explanation for the effects of ethephon-IBA combination in this study. It is possible that ethylene promoted IBA-mediated rooting from post-differentiated organs by activated ARFs, whereas IAA or anthranilic acid (a precursor of IAA) could not restore the reduction of synergistic effects owing to the fact that STS inhibits ethylene-activated ARFs.

We have demonstrated here that rooting abilities of *P. thunbergii* cuttings are dependent on genetic factors in donor plants, and that lower rooting abilities of cuttings from difficult-to-root donor plants are alleviated by the combinational treatment with ethephon followed by IBA. Using this combinational treatment, the cutting propagation of pine wilt-resistant plants with various rooting potentials will be more efficient.

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Fig. 1 Rooting abilities of cuttings from 31 seedlings within a half-sib family. Rooting abilities were evaluated as the percentage of rooted cuttings from 10 planted cuttings per donor plant

Fig. 2 Correlation between rooting abilities of cuttings from ortets and ramets. Eight to ten cuttings were planted from each donor plant and rooting abilities were evaluated per donor plant. Percentage data were transformed by arcsine. *r*-value with a *double asterisk* indicates a coefficient of correlation ($P < 0.01$)

Fig. 3 Rooting abilities (**a**) and number of adventitious roots (**b**) of cuttings soaked in Oxyberon (19.7 mM IBA) for three different periods of time. Ten cuttings were planted from each donor plant, and rooting abilities were evaluated per donor plant. Values are means ± SE of six donor plants (**a**) and those of all rooted cuttings (**b**). Significant differences are indicated by *different letters* ($P < 0.01$)

Fig. 4 Effect of Ethrel treatment on rooting abilities of cuttings. Ten cuttings were planted from each donor plant and rooting abilities were evaluated per donor plant. Values are means ± SE of 10 donor plants. Significant differences are indicated by *different letters* ($P < 0.05$). *Water* soaked in distilled water for 24 h; *ETH* soaked in 10,000-fold dilutions of Ethrel (69.2 µM ethephon) for 24 h; *IBA* soaked in Oxyberon (19.7 mM IBA) for 10 min as positive control

Fig. 5 Effects of combinational treatment with Oxyberon and Ethrel on rooting abilities of cuttings from difficult-to-root (**a**) and easy-to-root donor plants (**b**). Ten cuttings were planted from each donor plant and rooting abilities were evaluated per donor plant. Values are means ± SE of 10 donor plants (**a**) and those of three donor plants (**b**). Significant differences are indicated by *different letters* ($P < 0.05$). *ETH→IBA* soaked in 10,000-fold dilutions of Ethrel for 24 h before Oxyberon for 10 min; *IBA→ETH* soaked in Oxyberon for 10 min before 10,000-fold dilutions of Ethrel for 24 h; *IBA alone* soaked in Oxyberon for 10 min as positive control

Fig. 6 Effect of combinational treatment with authentic ethephon and IBA on rooting abilities of cuttings from difficult-to-root donor plants. Ten cuttings were planted from each donor plant and rooting abilities were evaluated per donor plant. Values are means ± SE of 10 donor plants. Significant differences are indicated by *different letters* ($P < 0.05$). *ETH→IBA* soaked in 69.2 μM ethephon for 24 h before 19.7 mM IBA for 10 min; *IBA→ETH* soaked in 19.7 mM IBA for 10 min before 69.2 μM ethephon for 24 h; *IBA alone* soaked in 19.7 mM IBA for 10 min as positive control

Fig. 7 Effect of STS, IAA and anthranilic acid on rooting abilities of cuttings with combinational-treatment (ethephon and IBA). Ten cuttings were planted from each donor plant and rooting abilities were evaluated per donor plant. Values are means ± SE of 13 donor plants. Significant differences are indicated by *different letters* ($P < 0.05$). *ETH→IBA* soaked in 69.2 μM ethephon for 24 h before 19.7 mM IBA for 10 min; *ETH+STS→IBA* soaked in a mixture of 69.2 μM ethephon and 100 μM STS for 24 h before 19.7 mM IBA for 10 min; *ETH+STS+IAA→IBA* soaked in a complex mixture of 69.2 μM ethephon, 100 μM STS and 10 μM IAA for 24 h before 19.7 mM IBA for 10 min; *ETH+STS+ANT→IBA* soaked in a complex mixture of 69.2 μM ethephon, 100 μM STS and 10 μM anthranilic acid for 24 h before 19.7 mM IBA for 10 min; *IAA→IBA* soaked in 10 μM IAA for 24 h before 19.7 mM IBA for 10 min; *IBA alone* soaked in 19.7 mM IBA for 10 min as positive control

Figure 1

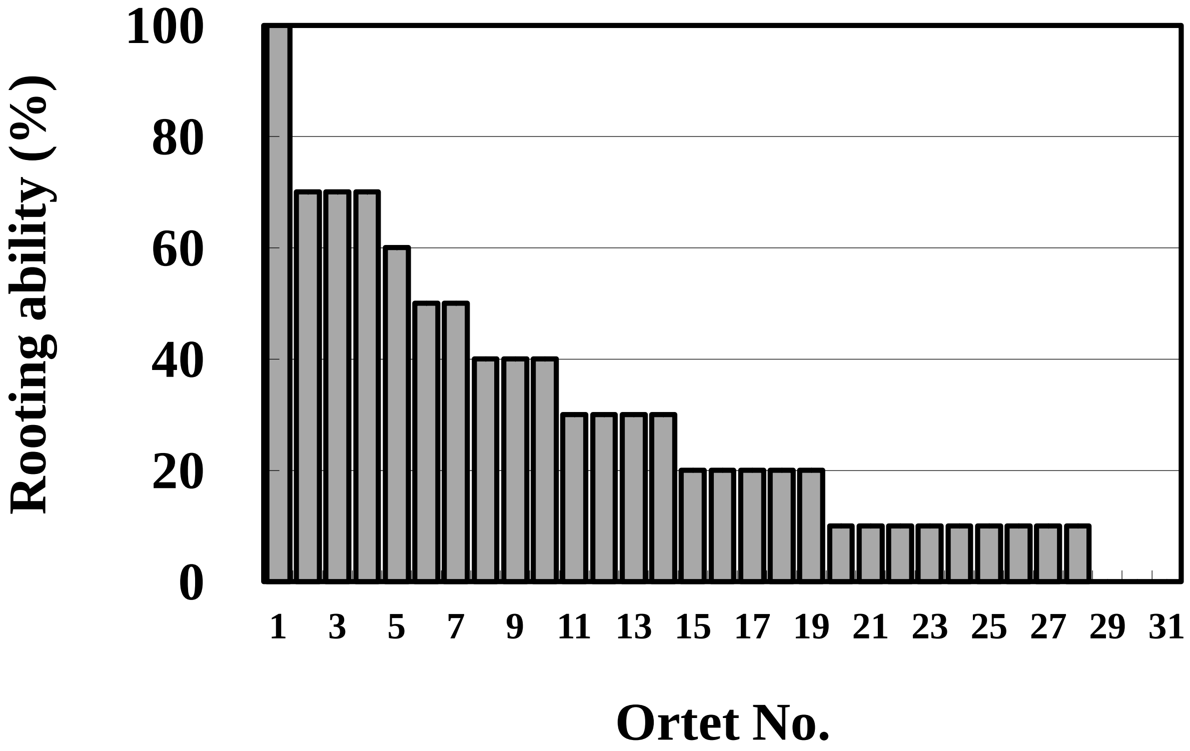

Rooting ability (%)
100
80
60
40
20
0
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
31
Ortet No.

Figure 2

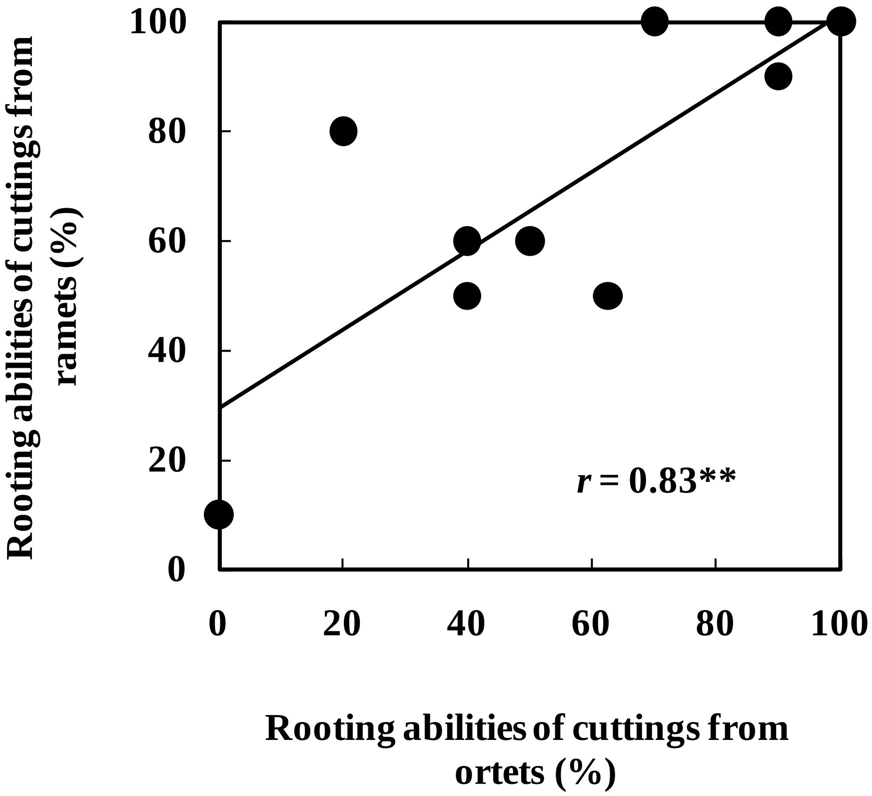

Rooting abilities of cutting from ramets (%)
100
80
60
40
20
0
r = 0.83**
0
20
40
60
80
100
Rooting abilities of cuttings from ortets (%)

Figure 3

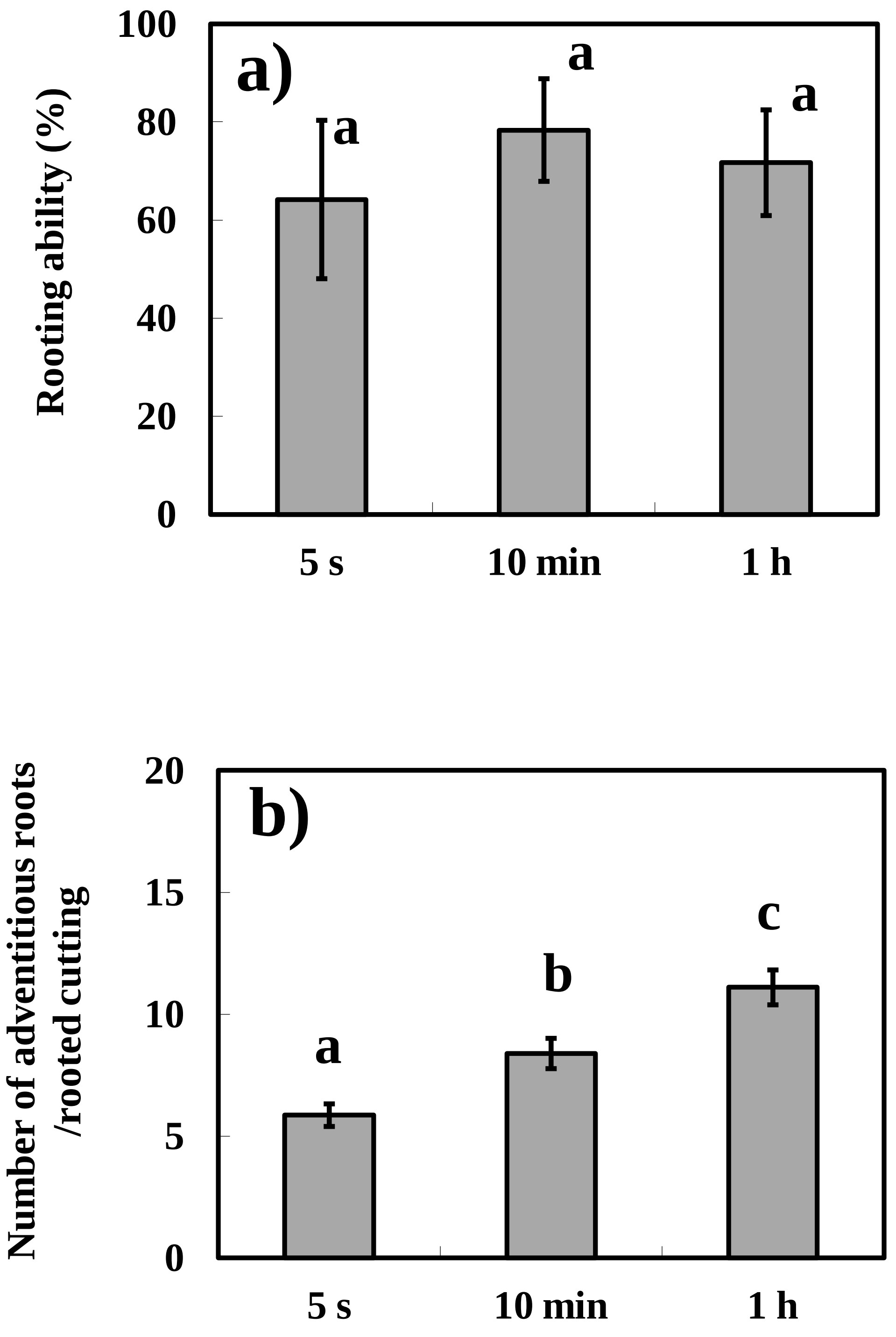

a)
Rooting ability (%)
100
80
60
40
20
0
a
a
a
5 s
10 min
1 h
b)
Number of adventitious roots /rooted cutting
20
15
10
5
0
a
b
c
5 s
10 min
1 h

Figure 4

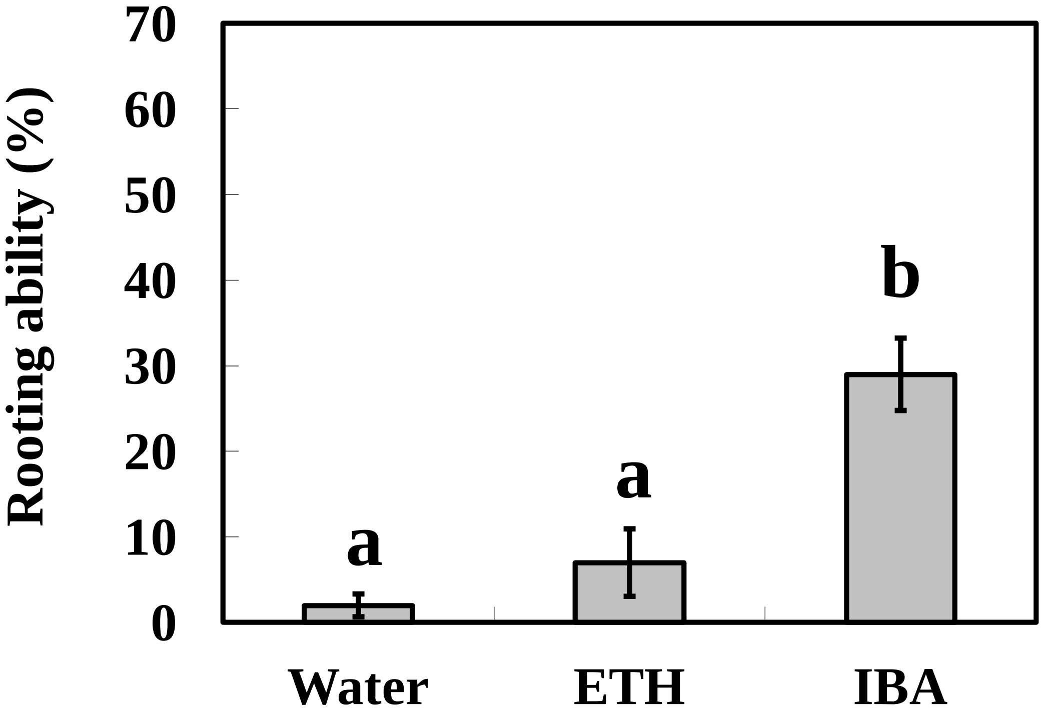
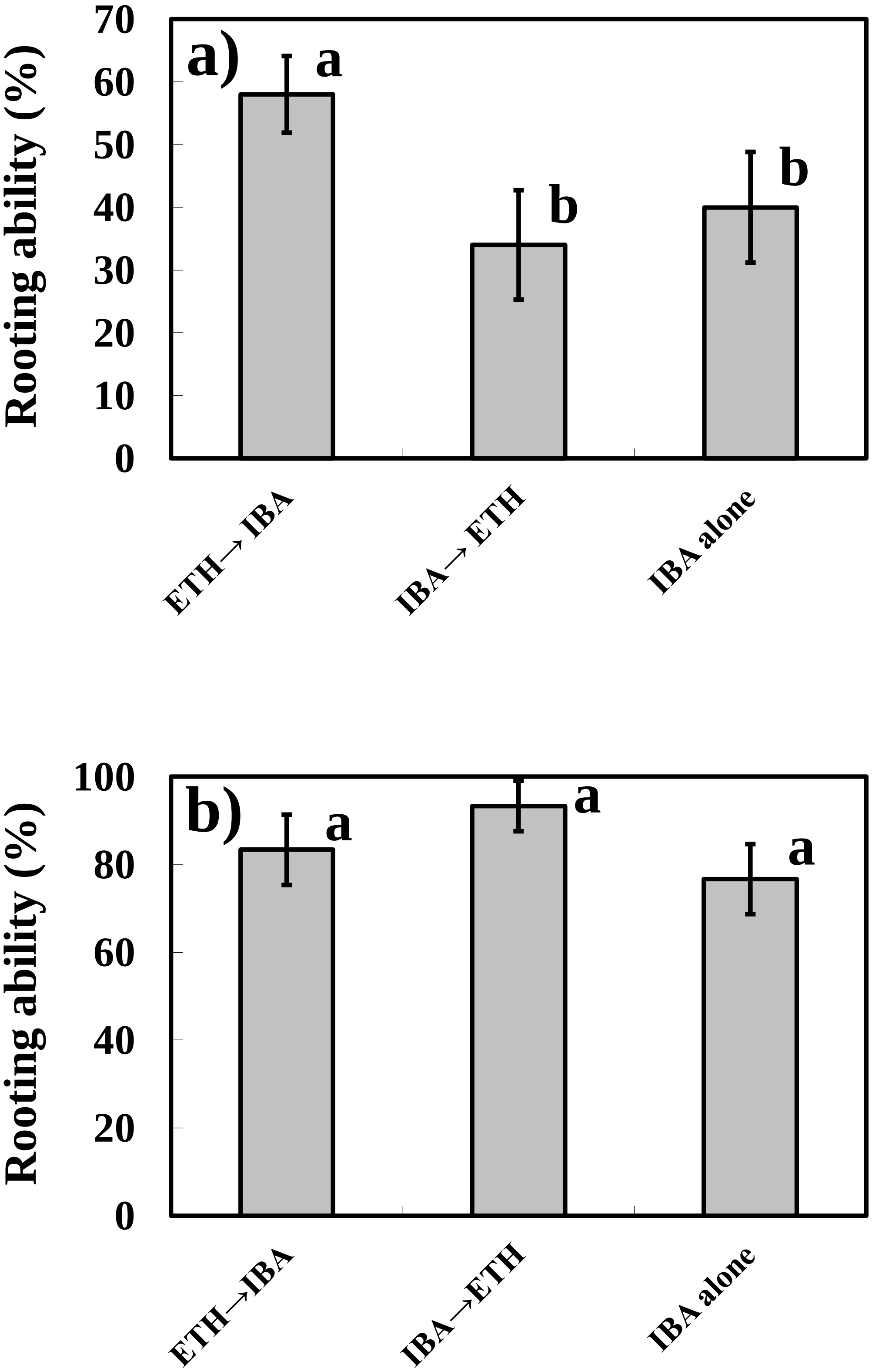


Figure 5



a)
Rooting ability (%)
0
10
20
30
40
50
60
70
a
b
b
ETH→ IBA
IBA→ ETH
IBA alone
b)
Rooting ability (%)
0
20
40
60
80
100
a
a
a
ETH→IBA
IBA→ETH
IBA alone

Figure 6

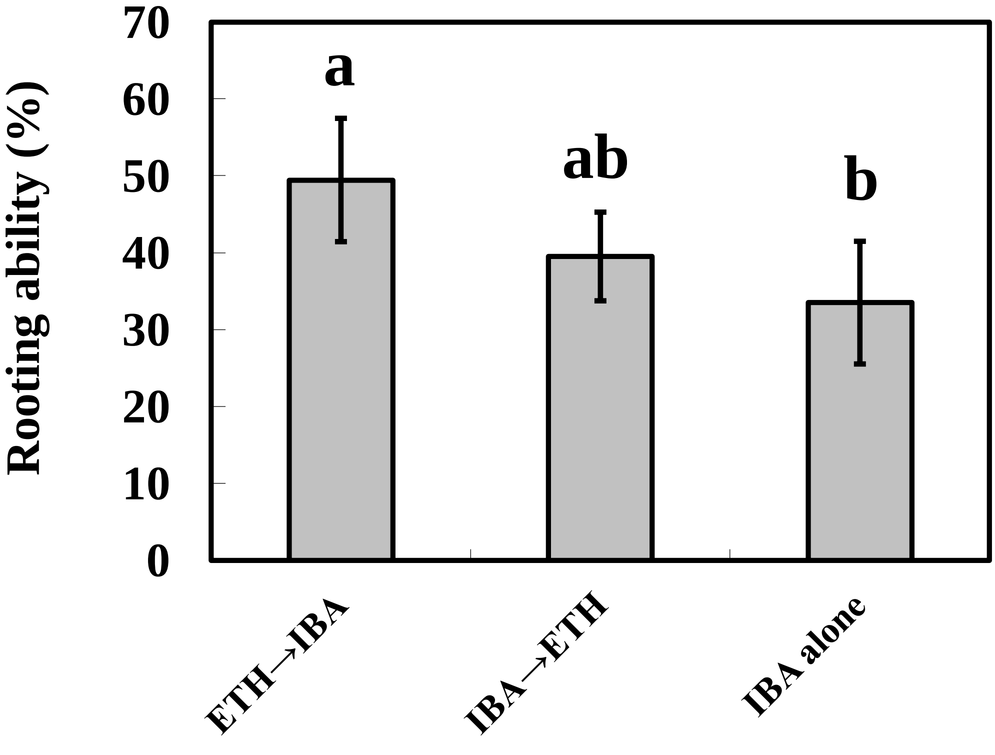
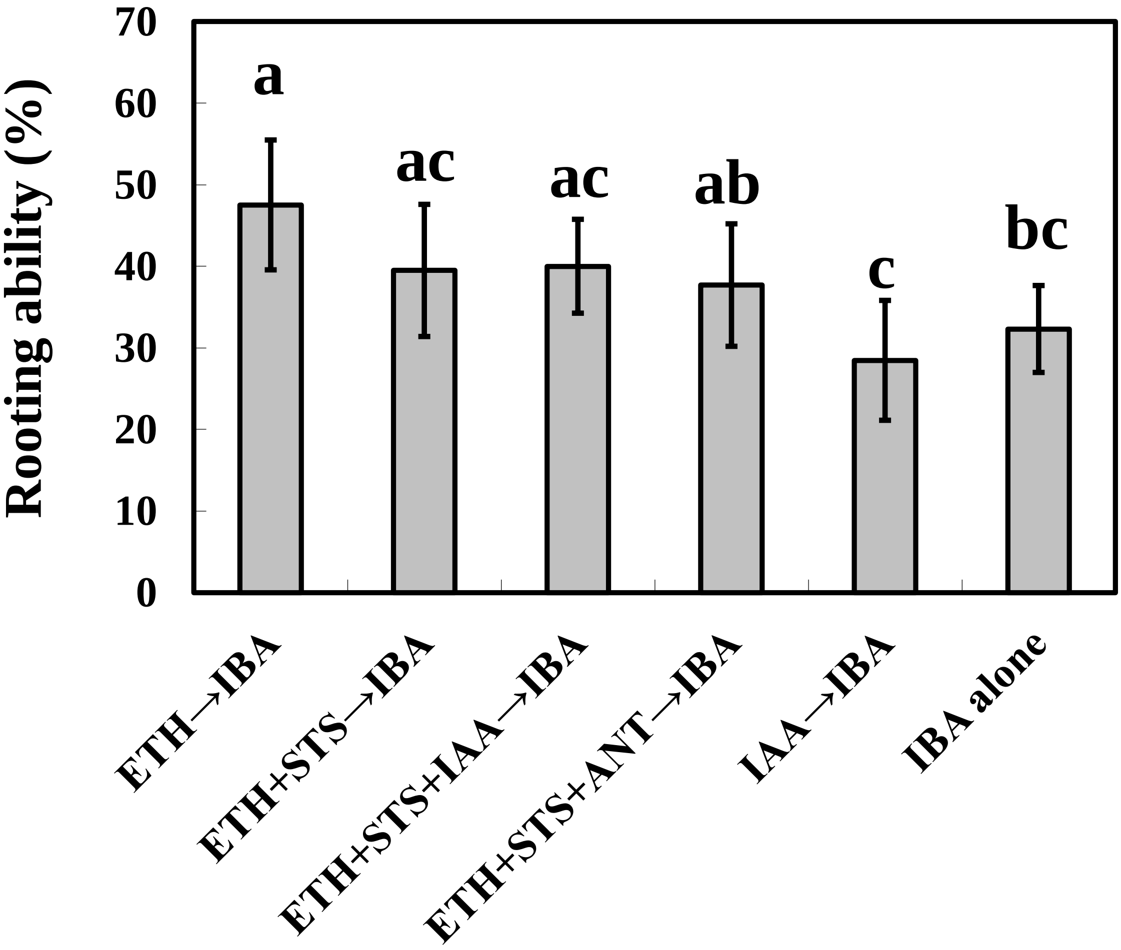

Rooting ability (%)
70
60
50
40
30
20
10
0
a
ab
b
ETH→IBA
IBA→ETH
IBA alone

Figure 7


Rooting ability (%)
0
10
20
30
40
50
60
70
a
ac
ac
ab
c
bc
ETH→IBA
ETH+STS→IBA
ETH+STS+IAA→IBA
ETH+STS+ANT→IBA
IAA→IBA
IBA alone