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Stress Enhances Indole-3-butyric Acid-induced Rooting in *Bupleurum falcatum* L. (Saiko) Root Culture

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Abstract

Indole-3-butyric acid (IBA) at 8 mg l^{-1} induced vigorous rooting in *Bupleurum falcatum* L. (Saiko) root culture in B5 medium after about 2 weeks, when it was added at the time when detached roots were transferred to fresh medium. The rooting activity markedly decreased with delay of IBA addition after root 2transplantation, and was hardly observed when IBA was added at the 7th day. Stresses such as drought or heat markedly promoted the IBA-induced rooting. Methylviologen, which causes superoxide generation in cells, and hydrogen peroxide also strongly promoted the rooting. We also examined the effects of active oxygen scavengers. Supplementation of some sugars, thiotaurin and methionine caused the rooting time delayed in a concentration-dependent manner. Histidine suppressed rooting much more, but did not the elongative growth of transferred roots at that time. All these results suggest that active oxygen species caused by stress are involved in the process of rooting with IBA.

Key words: active oxygen, auxin, *Bupleurum falcatum*, indolebutyric acid, root culture, rooting, Saiko, stress.

The root of *Bupleurum falcatum* (known as Saiko) is especially important in a variety of traditional Chinese medicines. It contains saikosaponins as the main active components, which possess anti-allergic, analgesic, anti-inflammatory, and other activities (Yamamoto *et al.*, 1975a, b; Kita *et al.*, 1980). In addition, saikosaponin-b_1 and -b_2, which are derived artificially from saikosaponin-a and -d, respectively, have potent anti-aging activity for skin (Nishiyama *et al.*, 1990). We have developed *B. falcatum* root culture as a mean of ensuring stable supply of high-quality Saiko extract (Kusakari *et al.*, 2000; Aoyagi *et al.*, 2001).

Auxins play a central role in lateral root and adventitious root formation (Thimann, 1936; Torrey, 1950; Gaspar and Hofinger, 1988). Exogenously applied auxins, such as indoleacetic acid, indolebutyric acid (IBA) and naphthaleneacetic acid, induce adventitious roots on stem cuttings of plants or root cutting (Hashimoto and Yamada, 1991). However, the action mechanism of auxins remains to be established.

On planting tree cuttings that do not readily generate roots, it is the empirical practice to expose the cuttings to various stresses, such as silver nitrate, permanganate, hot water and the like, in order to promote rooting. Kusakari *et al.* (2000) observed that lateral root formation on cultured roots of *B. falcatum* was clearly suppressed by sugar, which is a specific scavenger of hydroxyl radicals. In this paper, we present evidence that active oxygen species may be involved in IBA-induced rooting in *B. falcatum* root culture.

Induction and cultivation of adventitious roots of *B. falcatum* L. were described previously (Kusakari *et al.*, 2000). Adventitious roots were subcultured in B5 liquid medium (B5 medium) (Gamborg *et al.*, 1968) supplemented with 8 mg l^{-1} of IBA for 3 weeks and subsequently without IBA for 6 weeks at 23±2 ℃ on a gyrotory shaker (105 rpm) in the dark. Roots cultured for 42 days without IBA were used in experiments. IBA was sterilized by filtration with Millex GS filters (Nihon Millipore, Tokyo, Japan) before use. For experimental cultures, *B. falcatum* roots (0.2 g) were transferred into B5 medium (30 ml) containing IBA at 3, 4 or 8 mg l^{-1} (39 μM) in a 100 ml flask. Rooting started in about two weeks at 23±2 ℃. The rooting rate was evaluated at the third week by counting the number of roots generated in a region of 2 cm in the middle of primary roots under a stereomicroscope. In experiments on drought and heat stress, roots were cul-

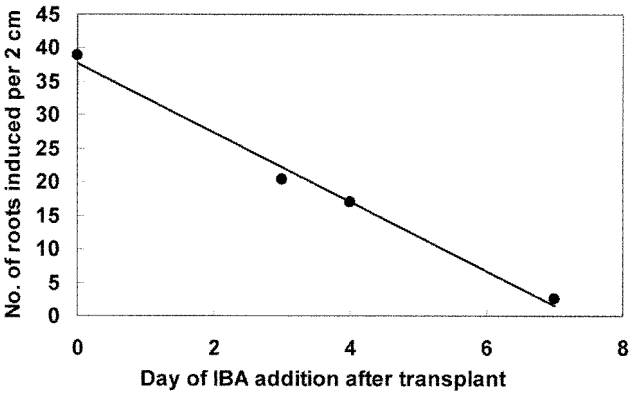

Fig. 1 Effect of timing of IBA addition on root generation. When the roots were transferred to the basal medium, IBA (8 mg l^{-1}) was added simultaneously, or thereafter at a designated time. The day when cultured roots were transplanted into fresh basal medium and incubation was initiated is designated as day 0. The number of roots generated in the middle part, 2 cm long, of the transferred roots was counted under a stereomicroscope after 3 weeks. Each value is the mean of 10 measurements. Three independent experiments gave similar results.

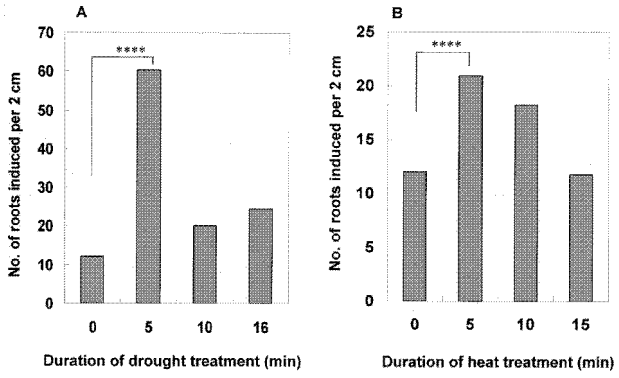

Fig. 2 Effects of drought or heat stress on root generation. The roots were cultivated in advance for 4 days in the basal medium. For drought stress (A), the roots were spread and kept for 5, 10 or 16 min on dry filter paper in a clean room, then transferred to fresh medium containing IBA (4 mg l^{-1}) for culture. To apply heat stress (B), the roots precultured for 4 days were transferred into fresh medium containing IBA (4 mg l^{-1}) and bathed in a water bath at 45 ℃ for 5, 10 or 15 min. The flask was cooled in running water and cultured thereafter. The number of roots generated was counted as described in the legend to **Fig. 1**. Each value is the mean of 19 measurments. ****$P<0.001$ for paired comparisons of stress-exposure for 5 min vs. control. Two independent experiments were done with similar results.

tured for 4 days in a basal medium before being used for experiments. To apply drought stress, the roots were spread and kept on a dry filter paper for 5-15 min in a clean room, then put in fresh medium containing 4 mg l^{-1} IBA, and culture was resumed. To apply heat stress, roots were immediately transferred into a fresh medium (4 mg l^{-1} IBA), and the flasks including roots were bathed in a water bath (45 ℃) for a designated time and thereafter cooled with tap water. Endogenous hydrogen peroxide was analyzed by the titanium method.

Auxin was essential to induce rooting in *B. falcatum* root culture. All auxins tested, IBA, indoleacetic acid, naphthaleneacetic acid, and 2,4-dichlorophenoxyacetic acid, induced rooting, although naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid inhibited elongation of the newly derived roots thereafter. IBA was the most efficient auxin for rooting and did not suppress elongation. IBA (8 mg l^{-1}) disappeared from the medium by the 4th day of culture. Even if the cultured roots were dosed with IBA for just the first 3 days, rooting occurred as vigorously as in the case when IBA was present in the medium throughout the experiment. We examined the effect of the timing of IBA addition on rooting (**Fig. 1**). When added together with roots to the medium at the starting time of culture, IBA (8 mg l^{-1}) induced vigorous rooting from the original roots after approximately 2 weeks. The rooting activity, however, decreased greatly as the timing of IBA addition was delayed, and was hardy expressed when IBA was added at the 7th day of culture. These results imply that some stress state in the root tissue, due to the detached roots being transferred to fresh medium, may be necessary for auxin to induce root generation. Therefore the effects of stress on rooting were examined. Cultured roots were exposed to stress in the form of drought or heat in the presence of IBA at suboptimal concentration (4 mg l^{-1}) after they had been cultured in basal medium for 4 days. Exposure to these stresses for 5 min strongly promoted the rooting induced by IBA (**Fig. 2**). We next examined whether active oxygen promoted rooting with IBA or not. Endogenous hydrogen peroxide level was analyzed in a pilot study; it was highest just after the roots were transferred to fresh medium and declined to a half in the first day, followed by a gradual decrease until new roots appeared (data not shown). The effects of exogenously added hydrogen peroxide on rooting were examined (**Fig. 3**). Rooting was hardly observed with 1 mg l^{-1} IBA, while it was prominently activated if hydrogen peroxide (5 mM) was supplemented. When methyl viologen (1 μM) was present only during the first 3 days,

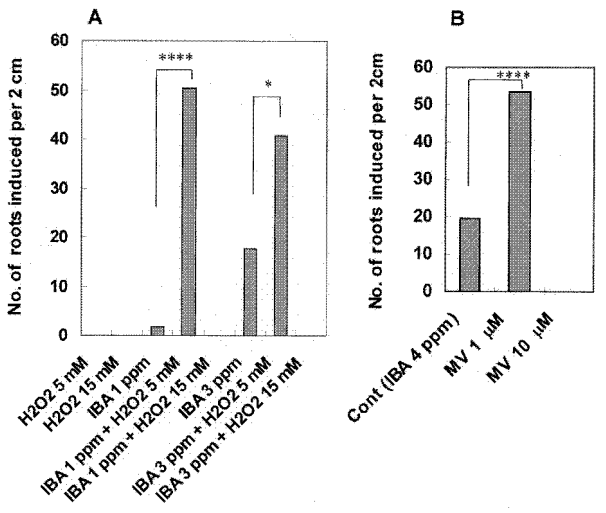

Fig. 3 Effects of hydrogen peroxide (A) and methylviologen (B) on IBA-induced rooting. Hydrogen peroxide was added simultaneously with IBA. Methylviologen (MV) was given with IBA (4 mg l^{-1}) only the first 3 days of the culture, then the roots was transferred to fresh B5 medium without MV and IBA, and culture was continued. The number of roots generated was counted as described in the legend to **Fig. 1**. Each value is the mean of 10 samples. ****P<0.001, *P<0.05 for paired comparisons of H_2O_2 (or MV) plus IBA vs. IBA only (control). Two experiments were done with similar results.

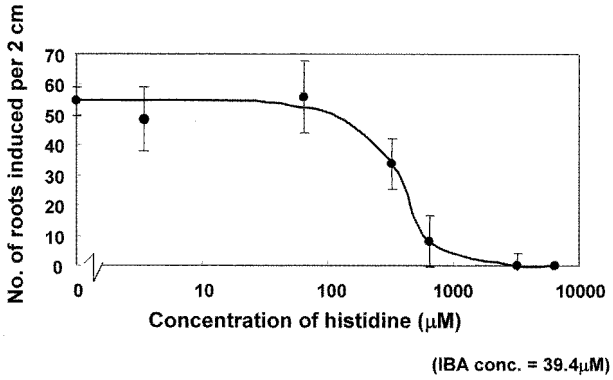

Fig. 4 Effects of histidine on rooting induction. Histidine was added simultaneously with IBA. The number of roots generated was counted as described in the legend to **Fig. 1**. Each value is the mean of 10 samples. Two independent experiments were done with similar results.

rooting was also clearly promoted over the control (4 mg l^{-1} IBA only) (**Fig. 3**). Methyl viologen was lethal, if kept in the flask throughout the culture.

As described above, active oxygen species such as hydrogen peroxide and superoxide can be positive factors promoting induction of new roots with IBA. Sucrose, which is known to act as a hydroxyl radical scavenger (Asada and Kiso, 1973), concentration-dependently suppressed rooting in Saiko root culture, but did not inhibit the growth of the roots after root generation (Kusakari *et al.*, 2000). We examined the effects of various sugars on rooting by observing the day when rooting started. When roots were cultured in B5 medium (3 mg l^{-1} IBA, 2% sucrose), rooting started at the 9th day. The rooting time was delayed by 2 days with supplementation of sucrose, glucose or 2-deoxyglucose (1% equivalent in medium, respectively) in addition to 2% sucrose. Mannitol (1% equivalent) completely inhibited the rooting; new roots did not appear at any point in the culture. Fructose or sorbitol (1% equivalent, respectively) did not show any clear effect. In a preliminary electron spin resonance experiment, glucose was more effective than fructose as a scavenger of hydroxyl radicals; fructose (10%) was equivalent to glucose (2%). Sucrose was intermediate in effectiveness. Sugars have multiple effects on rooting, for instance they can act as signal transducers (Rolland *et al.*, 2002). However, some of the above results could be at least partly due to the hydroxyl radical-scavenging activity. Catalase (0.1 and 1.0 mg ml^{-1}) was hardly effective when it was kept in the medium (including IBA) for the first 16 h, 3 days or throughout the experiment (data not shown). Next we examined the effects of singlet oxygen scavengers, such as histidine, thiotaurin and methionine on IBA-induced rooting. Histidine was examined in the concentration range from 0.0039 to 25.8 mM. It suppressed rooting at 0.3 mM or more, and was completely inhibitory at 3.2 mM (**Fig. 4**). We cultured the roots in medium containing IBA (8 mg l^{-1}) and histidine (0.4%, 25.8 mM) only during the first 3 days, and then transferred them to fresh basal medium without both IBA or histidine for 6 more weeks. A 3-day exposure period to IBA was sufficient for vigorous rooting, as described above. When histidine was also present in the medium during the same period, however, the rooting was completely abrogated (data not shown). Histidine at that concentration did not inhibit the elongative growth of transferred roots at all. When thiotaurin and methionine were added at 1, 2 or 4 mM to the medium (containing 8 mg l^{-1} IBA), the starting time of rooting was delayed in a concentration-dependent manner. Methionine suppressed even the swelling of the original transplanted roots, which is a common phenomenon in roots cultured with auxin. These data described above suggest that active oxygen is involved in IBA-induced rooting in Saiko root culture.

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