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# Efficient Plant Regeneration of *Ipomoea aquatica* by Direct Shoot Formation from Cotyledon Segments

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## Abstract

An efficient method for regeneration of *Ipomoea aquatica* was established. Cotyledons with petioles produced adventitious shoots near the excised margins situated only on the distal side, which rooted within one week in a 1/2 MS solid medium without phytohormones. The frequency of adventitious shoot formation depended on the developmental stage of the seedlings. Upon transfer to hydroponics in a greenhouse, all plantlets grew to maturation.

*Ipomoea aquatica* (pakbung) is a fast growing aquatic plant, a well-known vegetable in Southeast Asia of high nutritional value (Hashimoto *et al.*, 1985), which requires only two weeks of cultivation at an appropriate temperature after sowing for vegetable use. After decapitation of main stems, new shoots form from each stem nodule, making continuous harvest possible. Nitrogen and phosphorus absorption rates of this plant are as high as with the water hyacinth (Furukawa and Fujita, 1993). Since excess sulfur accumulation in soil and water is a serious problem in some locations near coalmines in Thailand, it has been suggested that this plant can be utilized for sulfur remediation by conferring rapid uptake and metabolism of exogenous sulfuric compounds (Ernst, 1998). Construction of genetically modified *I. aquatica* by introducing genes involved in sulfur assimilatory pathways is an interesting approach in this context. For this purpose, however, efficient regeneration and transformation systems are required. Here we report an efficient regeneration of *Ipomoea aquatica* through direct adventitious shoot formation from cotyledon segments.

## Plant materials and culture condition

Seeds of *Ipomoea aquatica* purchased from Takii Co. (Japan) were surface sterilized with 70 % ethanol for 2 min, 1 % NaOCl for 30 min, rinsed five times with sterilized distilled water, and sowed on a 30-m*l* 1/2 MS solid medium (Murashige and Skoog, 1962) supplemented with 30 g/l sucrose and 2.5 g/l gellan gum, pH 5.8 in a plastic Petri dish. After incubation at 25℃ under 16-h photo period at a light intensity of 50 $\mu$mol $m^{-2}s^{-1}$ (white fluorescent tubes), seedlings at the age of 4, 7, or 14 days were used as sources of explants. 1/2MS supplemented with 100 mg/*l* m-inositol, 0.1 mg/*l* thiamine-HCl, 0.5 mg/*l* folic acid, 30 g/*l* sucrose and 10 g/*l* agar, pH 5.8 (Mori *et al.*, 1999) was used as a basal medium throughout the experiment. For initial screening, various types of explants including cotyledons (cotyledon segments with or without petioles, petiole segments), cotyledonary nodes and hypocotyls from 7-day old seedlings were cultured on 1/2 MS solid medium containing 10 $\mu$M thidiazuron (TDZ). After 1 month, the frequency of adventitious shoot formation among 30 explants was examined. For more detailed analyses, cotyledon segments including petioles were excised from seedlings 4, 7 or 14 days after germination, and cultured on a modified MS solid medium containing 10 $\mu$M of 6-benzylaminopurine (BAP), isopentenyl adenine (iP) or TDZ in the presence or absence of 0.2 $\mu$M indole-3-butyric acid (IBA). Adventitious shoots of about 0.5-2 cm in length were detached from the parental explants and transferred to a 1/2 MS solid medium without phytohormones in a 300-m*l* square plastic bottle containing 50 m*l* of medium. Plantlets with shoots and roots were transferred onto a plastic mesh suspended on a 100 m*l* of 1/4 diluted MS liquid medium in a 300-m*l* square plastic bottle, which was capped for two weeks to allow plantlets to acclimatize before being exposed to glass house conditions. After 1 month, viability was estimated.

## Regeneration of mature plants

Cotyledons of *I. aquatica* seedlings feature a long petiole, the upper part of which is separated into two (**Fig. 1A**). For the preliminary test, cotyledons from 7-day old seedlings were cut into various sizes in different directions and cultured on 1/2 MS solid medium containing 10 $\mu$M TDZ. While hypocotyl segments produced only callus (**Fig. 1 B, C**), cotyledon segments bearing petioles produced adventitious shoots at as high a frequency as 75% (**Fig. 1 D, E**). Cotyledon segments without petiole or petiole segments alone did not form adventitious shoots. The results suggest the existence of a petiole-derived endogenous substance(s), whose distribution and accumulation may be important for polar development of adventitious shoots. In order to establish a regeneration method, the frequency of adventitious shoot formation was examined for cotyledons at different developmental stages in the 1/2 MS solid medium containing 10 $\mu$M TDZ, BAP or iP. The results showed TDZ to be the most effective growth regulator among the three cytokinins tested. The frequencies of direct adventitious shoot formation from cotyledon segments of 4, 7 and 14 days-old seedlings on medium containing 10 $\mu$M TDZ were 33, 75 and 3 %, respectively (**Fig. 2**). However, the frequency of adventitious shoot formation from seedlings at different developmental stages differed depending upon the kind of cytokinins used. TDZ was effective for 7 day-old explants, while BAP was effective for 14 day-old explants (**Fig. 2**). In the latter case, the cotyledon segments became brown within 14 to 21 days of culture, this being particularly conspicuous when explants originated from young seedlings, and such segments hardly produced adventitious shoots. In the presence of iP, the frequency of adventitious shoot formation was low at all developmental stages. However, growth of adventitious shoots was very fast and root formation from shoots was active.

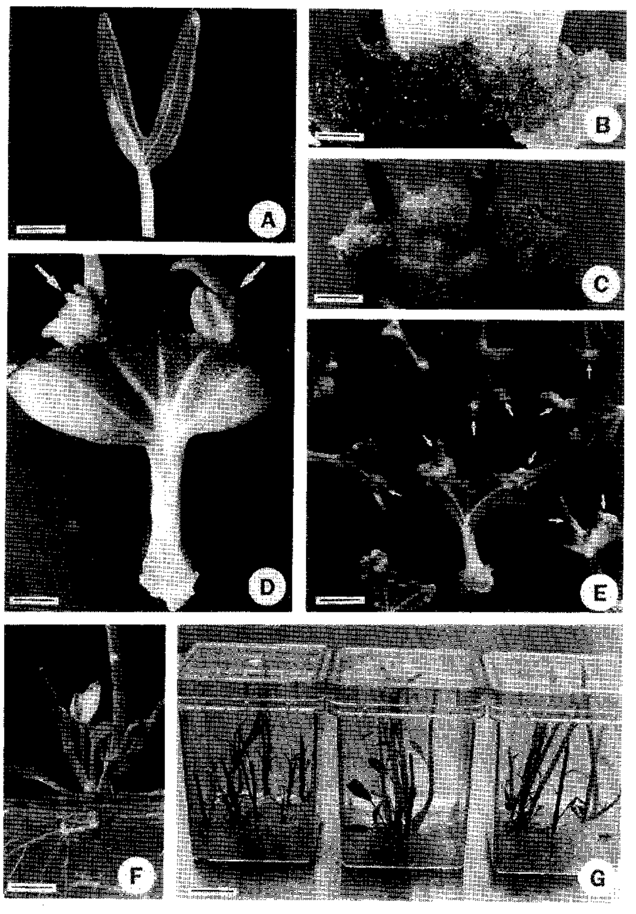


**Fig. 1.** Plant regeneration of *Ipomoea aquatica*. (A) Cotyledon of a 7 day-old seedling. (B) Callus formation from a cotyledon segment without a petiole on medium containing 10 $\mu$M TDZ after 1 month. (C) Callus formation and axillary shoot growth from a septum segment on medium containing 10 $\mu$M TDZ after 1 month. (D-E) Direct adventitious shoot formation (arrows) from cotyledon segments containing petioles on medium containing 10 $\mu$M TDZ. (F) Root formation at the base of shoots. (G) Plantlets with well-developed roots and shoots grown on a hormone-free medium after 1 month culture.

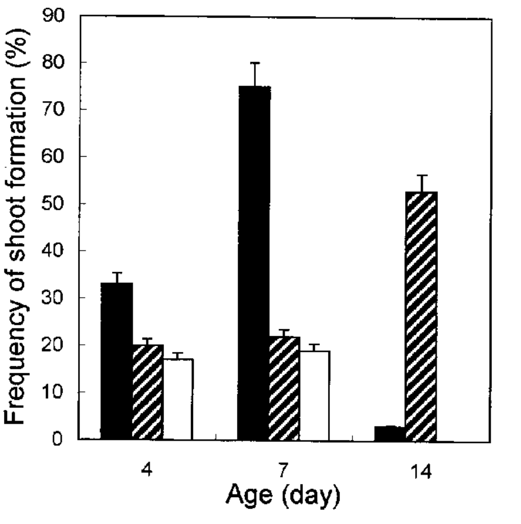


**Fig. 2** Frequency of shoot formation from cotyledon segments containing petioles from three stages of seedlings on medium containing 10 $\mu$M TDZ (solid bar), BAP (striped bar) or iP (open bar).

Combinations of IBA with all cytokinins tested resulted in frequencies of adventitious shoot formation less than 20 % at all developmental stages (data not shown), suggesting auxin to be not effective. The results indicate that adventitious shoot formation from cotyledon segments of *I. aquatica* is directly dependent on the developmental stage of the seedling and on the type of cytokinins. Cotyledon segments of 7 day-old seedlings and TDZ appeared to be the most suitable materials for their efficient induction. When small adventitious shoots with and without roots were detached from the parental explants and transferred to a 1/2 MS solid medium without phytohormones, further adventitious roots developed rapidly from their bases within a week (**Fig. 1 F**). Plantlets with well developed shoots and roots were transferred to 1/5 MS liquid solution, and after 2 weeks in a capped condition for acclimatization, they were exposed directly to green house conditions. All plantlets grew well to maturation (**Fig 1 G**).

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