HortScience 29(4):327-328. 1994.

PCIB Enhances Regeneration of *Ipomoea cordatotriloba* Dennstedt Leaf Explants and Protoplasts

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Additional index words. Ipomoea batatas, sweetpotato

Abstract. **Leaf callus of *Ipomoea cordatotriloba* was initiated by culturing explants on Linsmaier and Skoog medium containing 3 g yeast extract/liter, 18.9 μM ABA, 2.3 μM 2,4-D, and 0.15 M sucrose. Calluses were transferred to Murashige and Skoog media containing 17.8 μM BA and 0, 1, 10, or 100 μM PCIB. The number of shoots from calluses grown on medium containing 10 μM PCIB increased significantly, and the percentage of calluses exhibiting shoot regeneration almost doubled compared to calluses grown on regeneration medium without PCIB. Protoplasts isolated from stem and petiole tissues of in vitro-grown plants were cultured in Kao and Michayluk 8p medium to the callus stage. Calluses (4-6 mm) were transferred to the callus induction and regeneration media used to regenerate leaf-explant callus. Of the protoplast-derived calluses cultured on media containing 10 or 100 μM PCIB, ≈13% and 18%, respectively, regenerated shoots after 2 months; none regenerated on the medium without PCIB. Chemical names used: abscisic acid (ABA); 2,4-dichlorophenoxyacetic acid (2,4-D); N^6-benzyladenine (BA); α– p-chlorophenoxyisobutyric acid (PCIB).**

Sweetpotato *[Ipomoea batatas* (L.) Lam.] is the most important economic member of the section Batatas in the genus *Ipomoea*. It ranks third in worldwide root and tuber production with >11 million hectares producing >131 million tonnes (Food and Agriculture Organization of the United Nations, 1991).

Although genetic improvements in sweetpotato have been made without using related species (Jones, 1986), breeding progress may benefit, as it has in other crops, by incorporating wild germplasm (Kalloo, 1988). Sterility and self- and cross-incompatibility barriers found in the section Batatas have limited access to the genetic diversity of wild relatives (Martin, 1968, 1970a, 1970b). In vitro techniques, such as embryo rescue (Kobayashi et al., 1993; Mukherjee et al., 1991) and somatic hybridization, should facilitate the improvement of sweetpotato using incompatible germplasm. Because of the lack of suitable protoplast-regeneration systems, the exploitation of incompatible germplasm through somatic hybridization has not been accomplished yet.

Five of the 13 species in *Batatas* have been regenerated from explants: *I. batatas* (Carswell and Locy, 1984); *I. lacunosa* L. (Belarmino et al., 1992); *I. trifida* (H.B.K.) G. Don (Belarmino et al., 1992; Kobayashi, 1984); *I. triloba* L. (Liu et al., 1990); and *I. cordatotriloba* Dennstedt [corrected binomial for *I. trichocarpa* Elliott (Austin, 1988)] (Otani and Shimada, 1988). Only *I. triloba* obtained a high shoot regeneration rate from explant and protoplast callus (Liu et al., 1990, 1991). Shoots of the other four species were obtained primarily through regeneration of roots from callus and subsequent adventitious formation of shoots on the regenerated *(de novo)* roots and not indirectly from callus.

It is well known that the auxin : cytokinin ratio plays a major role in regeneration (Skoog and Miller, 1957). The tendency of *Ipomoea* species to regenerate roots and not shoots, even with low or no auxin in the medium (Kobayashi, 1992), may indicate high levels of endogenous auxin activity in the callus. PCIB has exhibited antiauxin activity in growth regulator studies using diverse crop species (Frenkel and Haard, 1973; Jacobs and Hertel, 1978; Quattrocchio et al., 1986; Trebitsh and Riov, 1987). Therefore, PCIB might help to reduce rooting and enhance shoot regeneration from callus in some *Ipomoea* species. Our report describes how PCIB affects shoot regeneration of *I. cordatotriloba* from leaf and protoplasm callus.

Materials and Methods

Ipomoea cordatotriloba (accession no. 62.19) seeds were obtained from A. Jones (U.S. Dept. of Agriculture-Agricultural Research Service, Vegetable Laboratory, Charleston, S.C.). The seeds were scarified and surface disinfested with concentrated H_2SO_4 for 20 min, rinsed three times with sterile distilled water, and germinated on MS medium (Murashige and Skoog, 1962). In vitro cultures were maintained by monthly shoot tip subculture.

Leaf explants were cultured using the method described by Otani and Shimada (1988) with some modifications. We cultured five to seven leaf pieces on callus induction medium (20 ml) in polystyrene petri plates (110 × 15 mm). Leaf explants were prepared by cutting 10-mm^2 squares from the center sections of leaves, ensuring each explant contained the leaf midvein. The callus induction medium consisted of LS medium (Linsmaier and Skoog, 1965) containing 3 g yeast extract/liter, 18.9 μM ABA, 2.3 μM 2,4-D, 0.15 M sucrose (pH 5.8), solidified with 0.2% Phytagel (w/v; Sigma Chemical Co., St. Louis). To test the effects of PCIB, explants were transferred after 1 month on callus induction medium to four different MS-based regeneration media supplemented with 17.8 μM BA and 0, 1, 10, or 100 μM PCIB. The cultures were maintained under 12 h photoperiod provided by cool-white fluorescent lamps (45-60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 27C. The experiment was a completely randomized design with four replications. The data were pooled and analyzed using SAS's GLM procedure (SAS Institute, 1987). After 2 months on the regeneration media, calluses were transferred to growth-regulator-free MS medium for shoot development. The number of regenerating shoots was recorded after ≈4 weeks.

For protoplasm experiments, 1-cm shoot tips were excised from stock cultures and propagated on 100 ml MS medium in PlantCon containers (ICN Biochemical, Costa Mesa, Calif.) maintained under a 12-h photoperiod provided by cool-white fluorescent lamps (45–60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 27C. Protoplasts were isolated from the petiole and stem tissues of 4-week-old shoots using methods described by Sihachakr and Ducreux (1987) with some modifications. The tissues were soaked in CPW salt solution (Frearson et al., 1973) overnight at 8C; then they were placed in enzyme solution (0.5–1 g tissue/10 ml) containing 1.5% (w/v) Cellulase Onozuka R-10 (Yakult Pharmaceutical Industries, Nishinomiya, Japan), 0.5% (w/v) Macerozyme R-10, CPW salts, 0.56 M sucrose, and 2.6 mM 2-[*N*-morpholino]ethanesulfonic acid (MES). The tissues digested for 8–12 h in darkness at 27C on a rotary shaker (75 rpm).

Initially, isolated protoplasts were cultured for 7 days in darkness in polystyrene petri plates at a density of 5×10^4 protoplasts/ml in KM8p liquid medium (Kao and Michayluk, 1975), containing 2.6 mM MES, 0.9 μM 2,4-D, 2.3 μM zeatin, and 2.7 μM α–naphthaleneacetic acid (NAA). Then the cultures were illuminated for 12 h daily with cool-white fluorescent lamps (60 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 27C. After 2 to 3 weeks, microcalluses (>1 mm in diameter) were transferred onto KM8p medium solidified with 0.4% (w/v) Seaplaque agarose (FMC, Rockland, Maine). After ≈2 months on solidified KM8p, yellow-green to light-green cal-

Received for publication 2 May 1993. Accepted for publication 16 Oct. 1993. Scientific article no. A6530, contribution no. 8738 of the Maryland Agricultural Experiment Station. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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luses (4 to 6 mm in diameter) were transferred to callus induction medium (Otani and Shimada, 1988). One month later, calluses were transferred to three regeneration media consisting of MS medium supplemented with 17.8 μM BA and 0, 10, or 100 μM PCIB. The experiment used a completely randomized design with three replications. After ≈6 weeks on the regeneration media, calluses were transferred to growth-regulator-free MS medium.

Results and Discussion

Callus from leaf explants that formed on the induction medium appeared soft, yellow, and friable. On all four regeneration media, calluses became green and compact, and roots were visible. Shoots appeared to regenerate from dark-green bud-like zones of organized growth 4–6 weeks after being transferred to regeneration media. Shoots developed into normal-looking plantlets on subsequent transfer to growth-regulator-free MS medium. Percentage of calluses forming on shoots almost doubled, and the average number of shoots per callus, from the calluses grown on medium supplemented with 10 μM PCIB, increased significantly compared to the calluses grown on media with 0 or 1 μM PCIB (Table 1). By adding 10 or 100 μM PCIB to the regeneration medium, an average of 7.9 and 5.3 shoots per callus, respectively, regenerated from leaf explant callus. In contrast, Otani and Shimada (1988) report regeneration mainly from *de novo* -formed roots with only a few shoots regenerating from callus.

A mean of $794 \pm 113 \times 10^3$ protoplasts/g of tissue (fresh weight) was isolated from stems and petioles. Cell division was observed as early as 3 days after isolation. Plating efficiency (percentage of the total number of cultured protoplasts that were dividing after 7 days) averaged 2.9% ± 0.7%. Compact, yellow-green to light-green calluses formed on solidified KM8p medium. On the callus induction and the three regeneration media, protoplast-derived calluses grew similarly to leaf-explant-derived calluses. After 6 weeks on the regeneration media, dark-green zones of organized growth appeared on protoplasm calluses, as was observed in the leaf-explant calluses. Eight shoots were regenerated from three of 23 (13%) calluses cultured on media containing 10 μM PCIB, and seven shoots were regenerated from six of 34 (18%) calluses cultured on media containing 100 μm PCIB. Shoot regeneration was absent on 21 calluses cultured on the regeneration medium without PCIB.

Adding PCIB to the regeneration medium increased shoot regeneration from callus but did not appear to decrease root formation on explant and protoplasm calluses. In general, fewer roots were observed on protoplasm calluses than on explant calluses. Roots regenerating from protoplasm- and leaf-explant-derived calluses remained short, growing no more than 2 to 3 mm long.

Otani and Shimada (1988) noted the formation of thick and thin root types in their study. We also observed two types of roots on protoplasm- and leaf-explant-derived calluses: thick root were ≥1 mm in diameter; thin roots were ≤0.5mm in diameter. Calluses grown on regeneration media containing PCIB appeared to have a greater proportion of thick roots to thin roots than those grown on the medium without PCIB. Among all the calluses, a few shoots developed adventitiously on thick roots after 1 month on growth-regulator-free MS medium; no shoots grew from thin roots. Shoots from the *de novo* roots emerged later than shoots regenerated from callus and were not included in the number of shoots listed in Table 1. Thick and thin root types have also been reported in *I. batatas, I. trifida,* and *I. triloba* (Belarmino et al., 1992; Carswell and Locy, 1984; Liu et al., 1990). Shoot formation also was detected on thick roots, but not on thin roots.

Ipomoea cordatotriloba organogenesis was enhanced by PCIB and occurred while the calluses were on regeneration medium, but many of the shoots produced on regeneration media did not develop fully into plantlets until calluses were transferred to the growth-regulator-free medium. PCIB appeared to be necessary for regeneration from protoplast-derived calluses. Regeneration from protoplasm calluses cultured on media without PCIB may occur, but at levels below those detected in this study.

Table 1. Average number of shoots per callus and percentage of calluses forming shoots of *Ipomoea cordatotriloba* Ace. no. 62.19 after 1 month on regeneration media containing 0, 1, 10, or 100 μM PCIB and 1 month on growth-regulator-free MS medium.

PCIB concn (μM)	Avg no. shoots/ callus (± SE)	Calluses forming shoots (%)
0	1.1 (0.5)	33
1	0.2 (0.1)	17
10	7.9 (2.7)	62
100	5.3 (1.5)	57

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