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Embryogenic Callus Formation from Immature Embryo of Japanese Morning Glory (*Pharbitis nil* Choisy)

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Development of an efficient plant regeneration from calli and cultured tissues remains an important step for genetic manipulation in the Japanese morning glory (*Pharbitis nil* Choisy). A few papers have been reported on tissue culture works ; plant regeneration from cultured tissues has been achieved utilizing shoot tips [1] and immature embryo [2, 3], and transgenic callus induced by infection of *Agrobacterium tumefaciens* [4]. However, in most cases, the plant regeneration frequency was very low and the genotypic difference in regeneration ability still remained.

Recently, significant progress has been achieved in embryogenic callus formation and genetic transformation in sweet potato (*Ipomoea batatas* (L.) Lam.), which belongs to *Convolvulaceae* family like the Japanese morning glory which is sometimes referred to as *Ipomoea nil*. Otani and Shimada [5] established an efficient method for embryogenic callus production from meristem tissues of sweet potato using altered plant growth regulators, 4-amino-3,5,6-trichlorophenoxyacetic acid (picloram), 3,6-dichloro-2-methoxybenzoic acid (dicamba) or 4-fluorophenoxyacetic acid (4FA) in the medium. Otani *et al.* [6] obtained several transgenic sweet potato plants by *Agrobacterium tumefaciens*-mediated transformation using the embryogenic callus. The present paper describes a simple, efficient and reproducible method for embryogenic callus production from the immature embryo of Japanese morning glory.

Two strains of Japanese morning glory, SU001 and Violet were used. Plants were grown in a greenhouse at 28°C. The immature fruits were harvested from donor plants at two to three weeks after flowering. The immature seeds of immature fruits were immersed in 70% (v/v) ethanol for 30 seconds and sterilized in 2% (v/v) sodium hypochlorite solution for 5 min, followed by rinsing three times with sterile water. After sterilization, immature embryos were excised from young seeds and were placed on LS medium [7] supplemented with several concentrations (1-2 mg/*l*) of different plant growth regulators (4FA, naphthaleneacetic acid (NAA) and picloram).

After 4 to 6 weeks of culture, immature embryos of SU001 inoculated on the media supplemented with 4FA or picloram started to form yellow and friable calli which produced somatic embryos (**Fig. 1**). The highest frequency of embryogenic callus formation (66.7%) was obtained on the medium supplemented with 1 mg/*l* 4FA (**Table 1**). The medium containing 2 mg/*l* picloram also stimulated embryogenic callus production, but its frequency was low (11.1%). On the other hand, no embryogenic calli formed from immature embyo of SU001 either on the plant growth regulator-free medium or the medium containing NAA, while direct somatic embryogenesis was observed at high frequencies on both media from the lower side of the hypocotyls (**Table 1**). Similar findings on the direct somatic embryogenesis have been reported by Yoneda and Nakamura [2] and Jia and Chua [3] on the media supplemented with or without NAA.

The embryogenic calli were only produced on the media supplemented with 4FA in Violet (**Table 2**). A higher concentration of 4FA (2 mg/*l*) was effective for the embryogenic callus formation in this strain. Direct somatic embryogenesis was also observed in this strain (**Fig. 2**).

In our preliminary study, embryogenic calli also

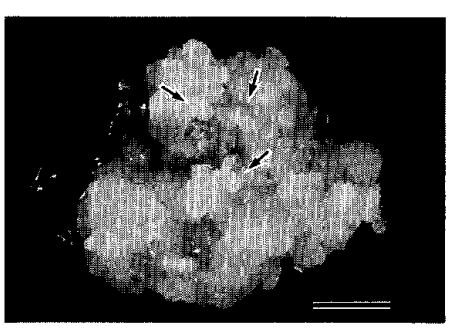

Fig. 1 Embryogenic callus from immature embryo of *P. nil* strain SU001 on the medium containing 1 mg/*l* 4FA. Arrows indicate somatic embryos. Bar=1 mm.

Table 1. Effect of plant growth regulators on embryogenic callus formation from immature embryo of *P. nil* cv. SU001.

Plant growth regulators*1	Concentration (mg/*l*)	No. of embryos cultured	No. of embryos producing an embryogenic callus (%)	No. of embryos producing a direct somatic embryo (%)
no	0	28	0	12(42.9)
4FA*2	1	30	20(66.7)	6(20.0)
4FA	2	35	15(42.9)	0
picloram	1	18	0	0
picloram	2	36	4(11.1)	6(16.7)
NAA*3	3	42	0	24(57.1)

*1 Basal medium was LS medium supplemented with 3% (w/v) sucrose and 0.32% (w/v) gellan gum.
*2 4FA : 4-fluorophenoxyacetic acid
*3 The concentration of sucrose was 6% (w/v) according to the method of Jia and Chua (1992).

Table 2. Effect of plant growth regulators on embryogenic callus formation from immature embryo of *P. nil* cv. Violet.

Plant growth regulators*1	Concentration (mg/*l*)	No. of embryos cultured	No. of embryos producing an embryogenic callus (%)	No. of embryos producing a direct somatic embryo (%)
no	0	20	0	3(15.0)
4FA*2	1	36	10(27.8)	1(2.8)
4FA	2	46	27(58.7)	3(6.5)
picloram	1	40	0	1(2.5)
picloram	2	27	0	0
NAA*3	3	28	0	18(64.3)

*1 Basal medium was LS medium supplemented with 3% (w/v) sucrose and 0.32% (w/v) gellan gum.
*2 4FA : 4-fluorophenoxyacetic acid
*3 The concentration of sucrose was 6% (w/v) according to the method of Jia and Chua (1992).

Table 3. Effect of immature embryo size on embryogenic callus formation in *P. nil* cv. SU001.

Size of embryo (mm)	No. of embryos cultured	No. of embryos producing an embryogenic callus (%)	No. of embryos producing a direct somatic embryo (%)
0.5-2.4	32	3(9.4)	14(43.8)
2.5-4.4	15	11(73.3)	3(20.0)
4.5-7.0	15	9(60.0)	3(20.0)

* Culture medium was LS medium supplemented with 1 mg/*l* 4FA, 3% (w/v) sucrose and 0.32% (w/v) gellan gum.

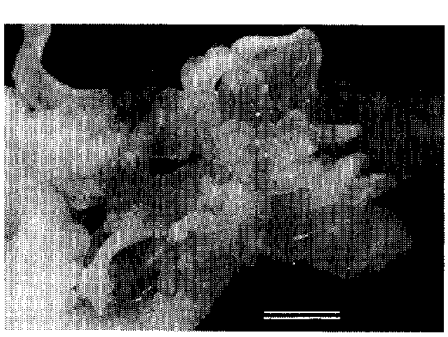

Fig. 2 Direct somatic embryo formation from immature embryo of *P. nil* strain Violet on the medium without plant growth regulators. Bar=1 mm.

formed from immature embryos of *P. nil*, strain Scarlett O'Hara and its related species, *P. purpurea*, strain IAC001 on the medium containing 1 mg/*l* 4FA or picloram (data not shown). These findings suggested that the altered plant growth regulators, 4FA and picloram were effective for embryogenic callus formation from immature embryos in the genus *Pharbitis*. Otani and Shimada [5] also reported that 4FA and picloram in the media were effective for embryogenic callus production in sweet potato. Therefore, 4FA and picloram might be effective plant growth regulators for embryogenic callus production in *Convolvulaceae* family.

As shown in **Table 3**, the size of immature embryos

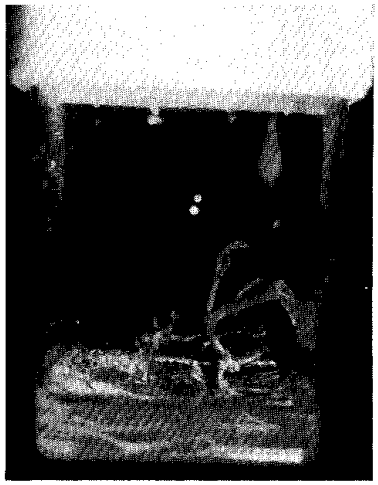

Fig. 3 Plant regeneration from embryogenic callus of *P. nil* strain SU001.

markedly affected embryogenic callus production in strain SU001. The embryos 2.5-4.4 mm in length gave the highest frequency (73.3%) of embryogenic callus formation, followed the largest embryos (4.5-7.0 in length) (60.0%) ; the smallest embryo (0.5-2.4 mm in length) gave the lowest frequency (9.4%) of embryogenic callus formation, but the highest frequency (43.8%) of direct somatic embryogenesis.

Numerous somatic embryos were formed from embryogenic calli transferred onto LS medium supplemented with 4 mg/*l* abscisic acid (ABA) and 1 mg/*l* gibberellic acid (GA_3). Somatic embryos germinated and developed into plantlets on LS medium containing 0.05 mg/*l* ABA (**Fig. 3**). These embryogenic calli proliferated by subculture onto fresh medium and maintained the ability to regenerate somatic embryos more than a year. The present culture system for embryogenic callus formation may be useful for the genetic transformation studies in Japanese morning glory.

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