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Short Communication

An efficient plant regeneration through embryogenic callus formation and direct somatic embryogenesis via immature embryo culture in *Ipomoea purpurea* and *I. tricolor*

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Abstract We established an efficient plant regeneration system to form embryogenic calli in common morning glory (*Ipomoea purpurea*) and blue morning glory (*I. tricolor*). Immature embryos of both morning glories cultured on media containing 1 mg l^{-1} 4-fluorophenoxyacetic acid (4FA) and 6% sucrose formed many embryogenic calli. The frequency of embryogenic callus formation was highest in *I. purpurea* strain Q74 (42.5%) and in *I. tricolor* cultivar 'Flying Saucers' (36.7%). Embryogenic callus formation differed with the genotypes in both morning glories. Numerous somatic embryos were formed from the embryogenic calli when the calli were transferred onto plant growth regulator-free medium.

Key words: Embryogenic callus, 4-fluorophenoxyacetic acid, *Ipomoea purpurea*, *Ipomoea tricolor*, plant regeneration.

Common morning glory (*Ipomoea purpurea*) and blue morning glory (*I. tricolor*) native to Mexico are widely cultivated as garden plant in Europe, America and Japan. These morning glories are now a popular a wall cover plant or "green curtain plant" which lowers the indoor temperature in an environment friendly way. There are some breeding issues, for example, yellow and orange flowers are difficult to produce. The new techniques in plant biotechnology such as protoplast fusion and genetic transformation are expected to enable further breeding of two ornamental *Ipomoea* species. While there are some reports on tissue culture (Jia and Chua 1992; Otani and Shimada 1998; Shimizu et al. 2003; Shimizu et al. 2005; Yoneda and Nakamura 1987) and transformation (Ono et al. 2000) in Japanese morning glory (*I. nil*), there are only one report on embryogenic callus formation in *I. purpurea* (Otani and Shimada 1998). Otani and Shimada (1998) reported that the possibility of embryogenic callus formation from immature embryo of *I. purpurea*, although they did not show any data for embryogenic callus formation and succeed in plant regeneration from embryogenic callus. And there are no reports on tissue culture and plant regeneration of *I. tricolor*. In the present study, we developed an efficient immature embryo culture method to regenerate *I. purpurea* and *I. tricolor*.

We used seven strains of *I. purpurea*, Q41, Q74, Q81, Q1123, 'Caprice', 'Light Blue Star (LBS)' and 'Pinky Shot', and three strains of *I. tricolor*, 'Flying Saucers', 'Heavenly Blue' and 'Blue Snow Storms (BSS)', a mutant derived from 'Flying Saucers' in this study. These plants were grown in the field at Ishikawa Prefectural University. The immature fruits were harvested about 2–3 weeks after flowering and surface-sterilized with 70% (v/v) ethanol for 30 s and then with a 3% (v/v) sodium hypochlorite solution containing a few drops of Tween 80 for 5 min. Then they were washed twice in a large volume of sterilized water and the immature embryos were excised. The immature embryo of *I. purpurea* was 2–7 mm and that of *I. tricolor* was 1–11 mm in length. The excised immature embryos were placed on LS medium (Linsmaier and Skoog 1965) supplemented with 1.0 mg l^{-1} 4-fluorophenoxyacetic acid (4FA) and 3% (w/v) sucrose (4F1) (Otani and Shimada 1998), 1.0 mg l^{-1} 4FA and 6% (w/v) sucrose (4F1S60), 1.0 mg l^{-1} picloram and 3% (w/v) sucrose (P1) (Otani and Shimada 1996), or 3.0 mg l^{-1} naphthaleneacetic acid (NAA) and 6% (w/v) sucrose (NAA3S60) (Jia and Chua 1992).

After four to ten weeks of culture, immature embryos of some strains inoculated on the media supplemented with 4FA started to form yellow and friable calli which produced somatic embryos (Figure 1a). The production of embryogenic callus (EC) in *I. purpurea* was observed in Q74, Q81, 'Caprice' and 'LBS' (Table 1), but not in the other three strains (Q41, Q1123 and 'Pinky Shot'), while non-embryogenic calli were formed in all strains. The ECs were produced only on the medium that contained

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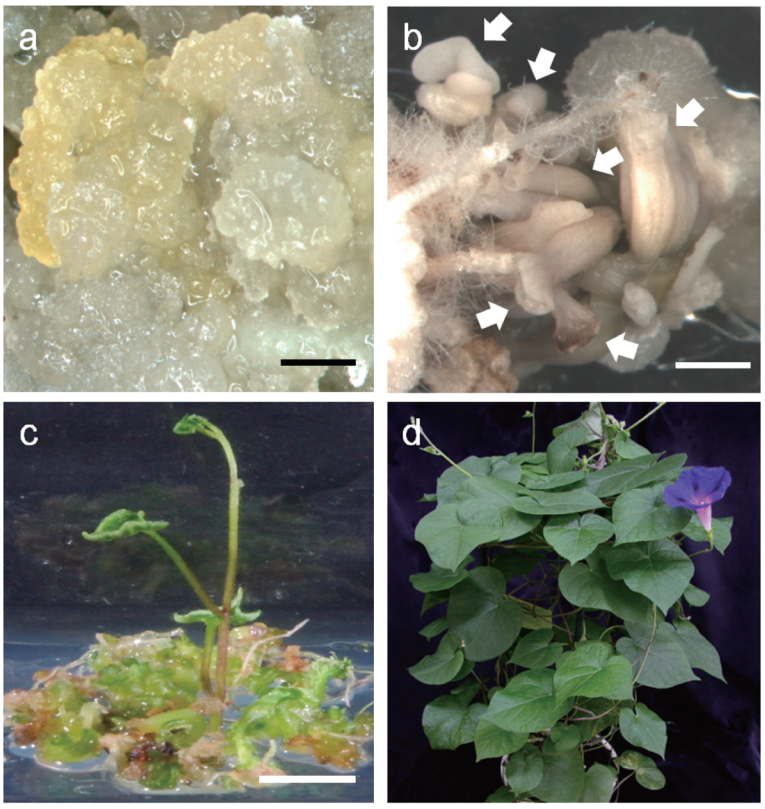

Figure 1. The immature embryo culture and plant regeneration from embryogenic callus (EC) of *I. purpurea* strain Q74. (a) The EC formed from immature embryo of Q74 cultured on 4F1S60 medium for 50 days. Bar=1 mm. (b) Direct somatic embryos formed from immature embryo of Q74 cultured on NAA3S60 medium for 50 days. Arrows indicates somatic embryos. Bar=1 mm. (c) Plant regeneration from EC of Q74 after two months of transfer to LS medium without plant growth regulator. Bar=1 cm. (d) Regenerated plant from EC of Q74 after 20 days of acclimatization.

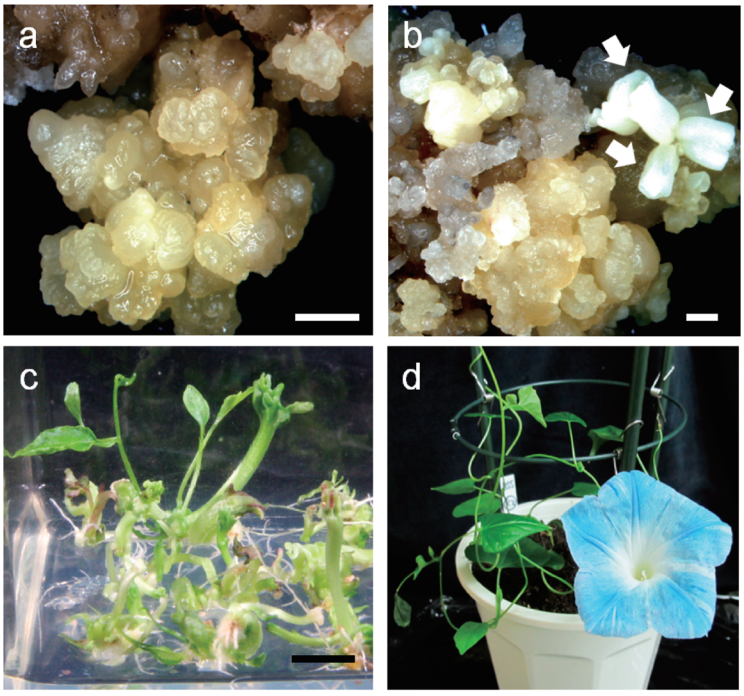

Figure 2. The embryogenic callus (EC) formation and plant regeneration from EC of *I. tricolor*. (a) The EC formed from immature embryo of 'Blue Snow Storms (BSS)' cultured on 4F1S60 medium for 70 days. Bar=1 mm. (b) Somatic embryo formation from EC of 'Flying Saucers' cultured on 4F1 medium for 70 days. Arrows indicates somatic embryos. Bar=1 mm. (c) Plant regeneration from EC of 'BSS' after two months of transfer to LS medium without plant growth regulator. Bar=1 cm. (d) Regenerated plant from EC of 'BSS' after 20 days of acclimatization.

Table 1. Frequency of embryogenic callus and direct somatic embryo formation from immature embryo of *I. purpurea*.

Cultivar	Medium name*[1]	No. of embryos cultured	No. of embryos producing embryogenic callus (%)	No. of embryos producing direct somatic embryo (%)
Q41	4F1	20	0	0
	4F1S60	20	0	4 (20±10)
	NAA3S60	20	0	7 (35±5)
	P1	—*[2]	—*[2]	—*[2]
Q74	4F1	30	7 (23.3±3.3)	5 (16.7±8.8)
	4F1S60	40	17 (42.5±11.8)	6 (15±6.5)
	NAA3S60	30	0	21 (70±21.9)
	P1	40	0	1 (2.5±2.5)
Q81	4F1	40	14 (35±10.4)	3 (7.5±0)
	4F1S60	40	16 (40±10)	5 (12.5±2.5)
	NAA3S60	30	0	6 (20±10)
	P1	30	0	2 (6.7±3.3)
Q1123	4F1	20	0	1 (5±5)
	4F1S60	40	0	5 (12.5±4.8)
	NAA3S60	20	0	6 (30±0)
	P1	20	0	1 (5±5)
Caprice	4F1	30	1 (3.3±3.3)	4 (13.3±3.3)
	4F1S60	40	6 (15±5)	5 (12.5±7.5)
	NAA3S60	20	0	1 (5±5)
	P1	30	0	0
Light Blue Star	4F1	50	5 (10±3.2)	0
	4F1S60	40	8 (20±7.1)	0
	NAA3S60	40	0	4 (10±4.1)
	P1	20	0	0
Pinky Shot	4F1	20	0	0
	4F1S60	20	0	0
	NAA3S60	20	0	0
	P1	—*[2]	—*[2]	—*[2]

*[1] Culture media are LS medium (Linsmaier and Skoog 1965) supplemented with 1.0 mg l^{-1} 4-fluorophenoxyacetic acid (4FA) and 3% (w/v) sucrose (4F1) (Otani and Shimada 1998), or 1.0 mg l^{-1} 4FA and 6% (w/v) sucrose (4F1S60), or 3.0 mg l^{-1} naphthalene acetic acid (NAA) and 6% (w/v) sucrose (NAA3S60) (Jia and Chua 1992), or 1.0 mg l^{-1} picloram and 3% (w/v) sucrose (P1) (Otani and Shimada 1996). *[2] Not tested.

4FA in four strains of *I. purpurea*. These EC were formed 4–6 weeks after the initiation of culture. The highest formation rate of EC was 42.5% in Q74 cultured on 4F1S60 medium (Table 1). The color of these EC was pale yellow in 'Caprice' and 'LBS', and yellow in Q74 (Figure 1a) and Q81. 'Caprice', 'LBS' and Q74 produced soft and wet calli, but Q81 produced friable and compact calli. In addition, proliferation of EC was rapid in 'Caprice', 'LBS' and Q74, but slow in Q81. By repeating subculture of EC, we produced callus lines. EC were easy to maintain in the callus lines of Q74 and Q81. However, EC were difficult to maintain in 'Caprice' and 'LBS' because of change to non-embryogenic calli during repeated subculture. All EC of 'Caprice' became non-embryogenic callus during subculture. It was obvious that frequencies of EC formation, characters of EC, growth of EC and maintainability for embryogenesis of EC in *I. purpurea* had the genotypic differences. On the other hand, higher concentration of sucrose in a medium was effective for EC formation. To overcome this genotypic differences, it might be necessary to examine not only the conditions of plant growth regulators but also the carbon source in a medium.

Moreover, direct somatic embryo (DSE) formation was observed in six strains of *I. purpurea* excluding 'Pinky Shot'. The highest frequency of DSE formation was 70% in Q74 cultured on NAA3S60 medium (Figure 1b). DSE formation was observed in several strains of *I. purpurea* on NAA3S60 medium that was reported to be effective for DSE formation in *I. nil* by Jia and Chua (1992).

EC were produced in all strains of *I. tricolor*. It was clear that 4FA was also effective for EC production in *I. tricolor*. The highest formation rate was 36.7% in 'Flying Saucers' cultured on 4F1 medium and 35% in 4F1S60 medium (Table 2). These ECs began to form about 7–10 weeks after the start of culture. The EC of 'BSS' was pale yellow and granular (Figure 2a), but EC in 'Heavenly Blue' and 'Flying Saucers' were yellow and friable. Thus, the character of EC of 'Flying Saucers' was clearly different from that of its mutant strain 'BSS'. Moreover, proliferation of EC was fast in 'Heavenly Blue', but slow in 'Flying Saucers' and 'BSS'. A uniform callus line was formed by repeated subculture of EC.

Meanwhile, DSE were observed in three strains of *I. tricolor*. The frequency of DSE formation was relatively lower than that of EC formation in all strains, and

Table 2. Efficiency of embryogenic callus and direct somatic embryo formation from immature embryo of *I. tricolor*.

Cultivar	Medium name*[1]	No. of embryos cultured	No. of embryos producing embryogenic callus (%)	No. of embryos producing direct somatic embryo (%)
Blue Snow Storms	4F1	30	3 (10±5.8)	2 (6.7±6.7)
	4F1S60	30	6 (20±5.8)	3 (10±5.8)
	NAA3S60	30	0	0
	P1	40	0	0
Flying Saucers	4F1	30	11 (36.7±3.3)	4 (13.3±6.7)
	4F1S60	20	7 (35±5.0)	1 (5±5)
	NAA3S60	20	0	1 (5±5)
	P1	—*[2]	—*[2]	—*[2]
Heavenly Blue	4F1	30	1 (3.3±3.3)	0
	4F1S60	30	5 (16.6±6.7)	0
	NAA3S60	30	0	2 (6.7±3.3)
	P1	30	0	3 (10±5.8)

*[1] Culture media are LS medium (Linsmaier and Skoog 1965) supplemented with 1.0 mg l^{-1} 4-fluorophenoxyacetic acid (4FA) and 3% (w/v) sucrose (4F1) (Otani and Shimada 1998), or 1.0 mg l^{-1} 4FA and 6% (w/v) sucrose (4F1S60), or 3.0 mg l^{-1} naphthalene acetic acid (NAA) and 6% (w/v) sucrose (NAA3S60) (Jia and Chua 1992), or 1.0 mg l^{-1} picloram and 3% (w/v) sucrose (P1) (Otani and Shimada 1996). *[2] Not tested.

the highest formation frequency of DSE was 13.3% in 'Flying Saucers' cultured on 4F1 medium (Figure 2b). The P1 medium was not effective for EC and DSE formation in both *I. purpurea* and *I. tricolor*. EC were easy to maintain in the callus lines of 'Flying Saucers' and 'Heavenly Blue'. These results suggested that 4FA and a higher concentration of sucrose were appropriate for EC formation in *I. purpurea* and *I. tricolor*.

The EC was formed from hypocotyl tissues of immature embryos in *I. purpurea*, while direct somatic embryogenesis was occurred from cotyledon tissues. On the other hand, the cotyledon tissues of immature embryos had showed no response and then turned brown on the culture media in *I. tricolor*. Immature embryo culture of *I. nil* showed similar responses to *I. purpurea* of this study (Yoneda and Nakamura (1987), Otani and Shimada (1998)), this result suggested that *I. purpurea* was closely related with *I. nil* than *I. tricolor*.

The EC formed from immature embryos of *I. purpurea* and *I. tricolor* were transferred to LS medium without plant growth regulators (PGR) to induce plant regeneration. In *I. purpurea*, green spots were observed 3–5 days after transfer and somatic embryos were observed at 7–10 days. In Q74 and 'LBS', adventitious root formation from EC was observed 10 days after transfer. Repeated subculture on same fresh medium at one month intervals, resulted in plant formation at 50–60 days after transfer (Figure 1c). Although, plant regeneration was observed in Q74, Q81 and 'LBS', many regenerated plantlets were vitrified. In Q74, somatic embryos were formed from all nine EC after transfer to LS medium without PGR, and thirteen plantlets regenerated from seven out of nine EC. Although, nine out of thirteen regenerated plantlets were vitrified, the remaining four plantlets showed a normal phenotype. In *I. tricolor*, green spots were observed on EC 4–6 days after transfer to LS medium without PGR, and somatic embryos were observed about 10 days after transfer in all three strains. By repeated subculture on LS medium without PGR every month, shoots were regenerated within 2–3 months (Figure 2c). Most of regenerated plantlets of 'Heavenly Blue' and 'Flying Saucers' were vitrified, although, all of the regenerated plants of 'BSS' had a normal phenotype. In 'BSS', somatic embryos were formed from all six EC after transfer to LS medium. Eight plantlets regenerated from all EC. Normal plantlets were then transferred to fresh LS medium without PGR for further growth. Rooted plants were then potted and grown at 26°C under 14-h photoperiod in a growth chamber.

In Q74, flowering was observed after 90 days of acclimatization. In 'BSS', flowering was observed after 20 days of acclimatization. These regenerated plants of Q74 and 'BSS' set seeds in the same way as a donor plant (Figures 1d, 2d).

As described above, we succeeded in forming EC and DSE from immature embryos in *I. purpurea* and *I. tricolor* and regenerating from EC. This is the first report on plant regeneration in *I. purpurea* and *I. tricolor.* 4FA was effective for the production of EC in *I. purpurea* and *I. tricolor.* These findings suggested that 4FA was advantageous for producing EC in *Ipomoea* genus. A few reports have been reported on the effect of 4FA in the plant tissue culture except the *Ipomoea* genus (Brisibe et al. 1994; Kamada et al. 2011; Shao and Taira 1990). An effect of 4FA on tissue culture might be examined in various plant species. In addition, a higher concentration of sucrose tended to be useful for the EC formation in both *I. purpurea* and *I. tricolor.* Lu et al. (1983) also reported that higher concentrations of sucrose (6 and 12%) caused an increase in the frequency of embryogenic callus formation in maize. The concentration of carbon source in the culture medium might also affect EC formation.

There have been some reports on transformation of *I. nil* using direct somatic embryos (Kikuchi et al. 2005; Ono et al. 2000) and transformation of *I. trifida* (Kakeda et al. 2009). It will be possible to produce transgenic plants of *I. purpurea* and *I. tricolor* using EC or DSE. It is valuable to establish these tissue culture systems in order to promote the experiments using biotechnology such as transformation in the future.

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