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Research note

In vitro culture and plant regeneration of large flowered purslane

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Abstract

Culture conditions were established for callus induction from a range of *Portulaca grandiflora* Hook tissues. Rapidly growing calli were obtained on Murashige and Skoog medium with stem-, leaf- and sepal-derived explants. Plant regeneration via organogenesis was explant-origin dependent with hypocotyl tissues giving the highest shooting frequency. Light conditions, pH and carbon source had a pronounced effect on the percentage of explants regenerating buds and the number of buds formed. It was possible to establish stable regenerated plants in the glasshouse.

Abbreviations: BA - 6-benzyladenine, 2,4-D - 2,4-dichlorophenoxyacetic acid, NAA - 1 -naphthaleneacetic acid, IAA - indoleacetic acid, MS - Murashige and Skoog (1962) medium

Portulaca grandiflora produces alkaloid-type betalain pigments and since the genetics of petal colouration in *P. grandiflora* is known (Adachi *et al.*, 1985) this species represents an ideal material for genetic experimentation (Rossi-Hassani & Zryd, 1994). To date, callus cultures of *P. grandiflora* have been obtained only from stems (Enderess, 1976; Konar, 1978). Konar (1978) reported that *P. grandiflora* cell cultures showed relatively slow growth rates and organogenesis occurred infrequently. The aims of the present work were to

—improve *in vitro* culture methods for callus initiation and growth, thereby facilitating biochemical approaches to the study of betalain biosynthesis, and

—standardize on a controlled system for plant regeneration that would be crucial for the application of genetic transformation methods.

Seeds of large-flowered purslane from the phenotype Pgfj (deep yellow coloured flowers) were surface disinfected with 10% (w/v) sodium hypochlorite for 10 min, washed with sterile water and placed for germination and growth *in vitro*, on basal MS medium (Murashige & Skoog, 1962), solidified with 0.6% (w/v) Difco purified agar. All explants, including hypocotyl explants, were taken from these *in vitro* cultures. Callus initiation and maintenance medium consisted of MS medium supplemented with 2% (w/v) sucrose and 0.6% (w/v) Difco purified agar. For shoot regeneration, MS medium was used, supplemented with 3% sucrose (MSA) or 3% glucose (MSB). These media were supplemented with growth regulators as indicated in the text and adjusted to pH 5.8 prior to autoclaving at 120°C for 20 min. Culture media (25 ml) were dispensed into 90 mm diameter petri dishes and sealed with Parafilm. All experiments were replicated four times. Data were transformed using an arcsin $\sqrt{Y}/100$ transformation to meet normality, which is recommended for Analysis of Variance (ANOVA) (Steel & Torrie, 1980).

Callus induction was possible from several plant organs. Explants, 2-3 mm in length, were taken from 2-month-old *in vitro-grown* plants and were placed on solid MS medium supplemented with combinations of growth regulators. Twenty explants per petri dish were

Table 1. Effect of growth regulator concentration on the frequency of shoot formation in hypocotyl explants of large-flowered purslane after 3 weeks of incubation.

Basal medium	NAA (μM)	BA (μM)	No. of explants	Explants forming shoots (%)[z]	Mean number of shoots per regenerating explant ± SE[y]
MSA	2.5	5	26	39b	4.3 ± 0.3
	2.5	10	24	33b	6.2 ± 0.2
	2.5	15	25	0	0
	5.0	10	24	0	0
MSB	2.5	5	25	84a	9.7 ± 0.3
	2.5	10	24	11c	4.8 ± 0.2
	2.5	15	25	36b	5.4 ± 0.2
	5.0	10	25	0	0

[z]Means followed by the same letter are not significantly different ($p < 0.05$) as determined by Duncan Multiple Range Test on arcsin √(Y/100) transformed means.
[y]Standard error of the estimated values.

maintained under long day conditions (16-h light at 360 nmol $m^{-2}s^{-1}$ 3500 Ingelec fluorescent tubes, 27°C day / 21 °C night). Calli were visible within 2 weeks along the cut edges. Callus tissues were removed from the explants and subcultured at 3-week intervals onto the same media upon which they were initiated. Stem explants gave the most frequent and prolific callusing response. Growth regulators affected callus texture, e.g. a combination of 3 μM 2,4-D / 5 μM kinetin gave white friable callus, while a combination of 5 μM NAA / 10 μM BA favoured the formation of green compact callus. Direct root production was observed in sepal and leaf explants with 2 μM kinetin and 1 μM 2,4-D, but was less frequent with 1.5 μM IAA and 3 μM BA. During callus initiation, direct organogenesis was often observed in hypocotyl explants, infrequently for stem, sepal, root and leaf explants. Successful callus induction from petals required the combined use of four growth regulators (2 μM 2,4-D; 2 μM NAA; 2 μM kinetin and 2 μM BA) and the presence of 10-100 μM gallic acid, a flowering inhibitor. Callus induction from petal explants was slow, requiring a period of culture in excess of 2 months before the first responses were observed. Stem callus formed readily in the presence of 2,4-D alone at concentrations ranging from 1-5 μM. Similar responses were obtained with root explants treated with kinetin alone at concentrations ranging from 0.5-1 μM. The most prolific callus growth was achieved on MS medium using a combination of 2 μM, 2,4-D and 2 μM kinetin with stem explants, or 5 μM 2,4-D and 1 μM kinetin with root explants.

Since the aim of the investigation was to obtain as many independent plant regenerants as possible, as would be required in the context of *Agrobacterium*-mediated transformation of purslane, hypocotyl sectors would appear to be the explant of choice. Thus 10-days-oldhypocotyIs were transversly sectioned and 23-27 segments, 2-3 mm in length, were placed on 25 ml solid MS medium supplemented with both BA and NAA, in the presence of either sucrose (MSA medium) or glucose (MSB medium) at 3% (w/v), with a range of pH values. Culture was initally conducted in the dark before transfer to long day conditions as earlier for a further 2 weeks. Shoot development occurred along the cut edges of the hypocotyls and was visible within 10 days. Four to 10 regenerating buds were formed per hypocotyl explant (Table 1). After removal of the developing shoots, these explants could be subcultured for a further 3 weeks, permitting initiation of new shoots. Analysis of variance detected significant differences among treatments. A combination of 2.5 μM NAA and 5 μM BA in MSB medium gave the highest frequency of explants producing shoots and the most shoots per explant.

The initial pH of the culture medium also affected the frequency of shoot formation (Table 2). Significant differences were observed among treatments. The highest number of shoots/explant were obtained with initial pH values between 5.5 and 6.0. This response could be improved when glucose was used as the carbon source (MSB medium). A 1-week preculture period in complete darkness improved the rate of morpho-

Table 2. Effect of pH and dark/light treatment on shoot induction in hypocotyl explants taken from 10-day-old hypocotyls after 3 weeks of incubation.

Basal medium	pH	Time (weeks) in Dark	Time (weeks) in Light	Shoot formation at different BA concentration (μM)[z,y] 5	10	15
MSA[x]	4.1	-	-	42(23)c	4(22)h	4(25)h
	5.5	-	-	36(22)d	24(24)e	0(24)
	6.0	-		46(25)c	17(22)f	4(22)h
	6.8	-	-	27(24)e	5(23)h	0(22)
MSB[x]	4.1	_	_	4(22)h	0(24)	4(22)h
	5.5	-	-	64(24)b	8(23)g	12(25)g
	6.0	-	-	95(24)a	12(23)g	27(24)e
	6.8	-	-	27(23)e	2(24)h	14(22)g
MSA[w]		0	3	21(24)f	19(22)f	4(24)h
	-	1	2	46(25)c	0(24)	4(24)h
	-	2	1	10(24)g	0(22)	0(22)
	-	3	0	0(25)	0(24)	0(22)
MSB[w]		0	3	92(24)a	12(25)g	21(22)f
	-	1	2	25(23)e	5(24)h	21(23)f
	-	2	1	0(25)	0(22)	0(24)
	-	3	0	0(25)	0(24)	0(23)

[z]A11 media contained 2.5 μM NAA.
[y]Number of explants in parentheses.
[x]Cultures were started in darkness for 2 days and then explants were transferred to a 16-h photoperiod.
[w]Media were adjusted to pH 6.0 before autoclaving.
Means followed by the same letter are not significantly different ($p \leq 0.05$) as determined by Duncan Multiple Range Test on arcsin $\sqrt{(Y/100)}$ transformed means.

genesis for cultures maintained on MSA medium with initial pH 6.0. Cultures maintained on MSB medium required only 1 or 2 days of dark to obtain a similar response.

Regenerated shoots were transferred to MSB medium supplemented with 5 μM BA at pH 6.0, for a period of 1 week, under long-day conditions and then transferred to MS growth regulator-free medium, supplemented with *2%* (w/v) sucrose, for rooting prior to transfer to glasshouse conditions. No additional treatment was required for rooting, which occurred in all regenerated shoots, with a 100% survival rate of plants in the glasshouse. All plants were morphologically normal and fertile upon self- and cross-pollination. Root tips were collected from 10 regenerants and stained with mithramycine according to Galbraith *et al.* (1983). All analysed regenerants contained a normal diploid chromosome complement (2n - 2x = 18), which is important if potentially stable transgenic regenerants of *P. grandiflora* are to be obtained using this regeneration protocol.

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