Plant regeneration from protoplasts of *Dimorphotheca* and *Rudbeckia*

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**ABSTRACT**

Protoplasts were isolated from leaves, shoots, cotyledons, ray florets and callus cultures of *Dimorphotheca aurantiaca* (syn. *D. sinuata*) (Cape Marigold, Star of the Veldt) and *Rudbeckia hirta*, *R. laciniata* and *R. purpurea*; species of ornamental value. For *Dimorphotheca*, plants were regenerated from protoplasts of all sources apart from the ray floret, whilst for the *Rudbeckia* species, although protoplast division was induced in most cases, only leaf mesophyll protoplasts of *R. hirta* c.v. Marmalade gave plants. The establishment of plant regeneration for these ornamental species, from protoplasts, now provides a basis for their somatic hybridisation.

**ABBREVIATIONS**

BAP, 6-benzylaminopurine; IAA, indole-3-acetic acid; NAA, naphthaleneacetic acid; K, kinetin; GA_, gibberellic acid; MS, Murashige and Skoog (~962); f.wt., fresh weight.

**INTRODUCTION**

Plant regeneration from protoplasts has been described for members of several genera within the family Compositae including Gaillardia, Cichorium, Senecio (Binding et al., 1981, Crepy et al., 1982), Helianthus (Bohorova et al., 1986), *Chrysanthemum* (Otsuka, 1986), and *Lactuca* species (Brown et al., 1987). With the exception of *Chrysanthemum* there are few ornamental species where regeneration has been established yet the Compositae is recognized as containing a vast number of commercially important ornamentals. The establishment of plant regeneration from such species will progressively allow for their realistic incorporation into somatic hybridisation programmes designed, ultimately, to foster their improvement in relation to their floral and marketable qualities.

**MATERIALS AND METHODS**

**Plant Material**

**Dimorphotheca**

Seeds of *D. aurantiaca* (cvs. Giant Orange, Glistening White and 'New Hybrids', Suttons Seeds Ltd., Torquay, U.K.) were surface sterilised in 10% Domestos solution (Lever Bros., U.K.) (25 min) followed by six changes of sterile tap water and germinated, in the dark, on MS medium lacking growth regulators but with 0.8% agar (Sigma), 3% sucrose. Cotyledons, of 12-14 day old seedlings, were used for protoplast isolation. Plants were also grown in the greenhouse (6000 lux, 16h daylength, mercury vapour lights, 20-25°C) and leaves (3rd/4th leaves of 25-40 day old plants), stems and fully expanded ray florets taken for protoplast isolation. Materials were surfaced-sterilised as for seeds (15 min).

Leaf callus, for protoplast isolation, was established on MS medium with 2.0 mg/l IAA, 1.0 mg/l BAP, 3% sucrose, 0.8% agar (pH 5.8) (MSD~ medium (Power et al., 1984), and grown routinely at 23°C under continuous illumination (2000 lux, daylight fluorescent tubes) with subculturing every 3-4 weeks.

**Rudbeckia**

Seeds of *R. hirta* (cv. Marmalade), *R. laciniata* (cv. Irish Eyes) and *R. purpurea* (syn. *Echinacea purpurea*) (cv. Brilliant Star) (Suttons Seeds Ltd, Torquay, U.K.) were sterilised (50% Domestos, lh) with in vitro cultured seedlings and greenhouse plants maintained as for *Dimorphotheca*. Callus cultures were established on MS medium with 2.0 mg/l BAP, 1.0 mg/l IAA, 3% sucrose and 0.8% agar (pH 5.8).

**Protoplast isolation and culture:**

**Rudbeckia**

The lower epidermis of surface-sterilised leaves, cotyledons and ray florets was removed by peeling and tissues were plasmolysed, for 1h in CPW salts, (Pearson et al., 1973) with 13% mannitol for the ray florets or 5% mannitol for the others (pH 5.8) and transferred to an enzyme mixture which consisted of 0.8% Cellulase R10 (Kinki Yakult, Nishinomiya, Japan), 0.4% Driselase (Kyowa Hakko Kogyo Co., Tokyo, Japan), 0.8% Rhizyme HP 150 (Roehm and Hass, Philadelphia, U.S.A.), 0.2% Macerozyme R10 (Kinki Yakult), CPW salts 5% mannitol for the leaves and cotyledons, 13% mannitol for the ray florets (pH 5.8) with antibiotics, 400.0 mg/l ampicillin (Boots, Pure Drug Co. Ltd., Nottingham U.K.), 10.0 mg/l gentamycin, 10.0 mg/l tetracycline (Sigma). Leaves, cotyledons and ray florets were incubated at 23°C (3-4h) on a rotary shaker (30 cycles/min) followed by a stationary incubation in the dark (6-10h).

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Protoplasts were released from callus using the same enzyme mixture but with Macerozyme R10 at 0.4% (12h on a rotary shaker). After incubation tissues were passed through a nylon sieve (64 μm pore size), washed twice in CPW salts, with 8% mannitol for the leaves and cotyledons, 13% mannitol for the others, by resuspension and centrifugation (80 x g; 5 min) and finally freed of debris by floatation in CPW21S medium (CPW salts with 21% sucrose) coupled with centrifugation (100 x g; 5 min). Protoplasts were transferred to the appropriate culture medium (see Results) for 24h in the dark and subsequently diluted to a density of 2.5 x 10⁶/ml and plated as liquid cultures (4 ml per 5 cm Petri dish, A/S Nunc, Roskilde, Denmark), agarose bead cultures (Shillito et al., 1983) (0.2 ml beads surrounded by 2.0 ml of liquid medium (5 cm Petri dishes). Dishes were sealed with Nescofilm and maintained in the dark or with a continuous illumination of 700 lux (daylight fluorescent tubes) (23°C). Protoplast culture media were replaced every 5-7 days with the same medium before reduction of the osmotic pressure was initiated. The following three media regimes were used with a progressive reduction in the osmotic pressure (every 5 days) being initiated at 16 days for callus protoplasts and 30 days for the other systems by the replacement of the protoplast culture medium with medium of lower osmotic pressure: -

- **MSP19M medium** (MS medium with 2.0 mg/l NAA, 0.5 mg/l BAP, 9% mannitol, pH 5.8) diluted with MSP19M medium (as MSP19M but no mannitol) in the ratios (ml) 3:1, 2:2, 1:2, 0:4; **K8P medium** (Kao and Michayluk, 1975) with K8 and K8P medium with K8 (Kao, 1977), the latter two media systems being utilised as for MSP19M medium in terms of handling for dilution. Plating efficiency was assessed after 9 days.

Protoplast-derived calli were finally transferred to MS medium with 1.0 mg/l IAA, 2.0 mg/l BAP, 0.8% agar, 3% sucrose (pH 5.8). Shoots were rooted in MS medium with 0.00875 mg/l IAA, 0.03 mg/l K, 0.001 mg/l folic acid, 3% sucrose, 0.7% agar (pH 5.8) (BGS) medium (Power et al., 1984) whereupon they could be transferred to the open greenhouse.

**Dimorphotheca**

Leaves, cotyledons, ray florets and stem segments were plasmolysed for 1h in CPW salts (with 8% mannitol for leaves/cotyledons or 13% mannitol for stems/ray florets). Protoplasts were released from leaf/cotyledon tissues using an enzyme mixture which consisted of 2.0% Meicelase (Meiji Seika Kaisha Ltd, Japan), 2.0% Rhozyme HP 150, 0.03% Macerozyme R10, CPW salts, 8% mannitol (pH 5.8). For callus/stem/ray floret tissues the enzyme mixture was as for Rudbeckia callus.

Incubation (dark, 23°C) was static for leaves (5-6h), cotyledons (10-12h) and ray florets (10h), whilst for stem/callus tissues incubation was on a rotary shaker (4-5h, 30 cycles/min) followed by static conditions (8-10h). The purification and culture of protoplasts was as for Rudbeckia.

Protoplast derived calli (1.5-2.00 mm diam.) were transferred to MS medium (3-5 weeks) for proliferation and then to MS medium (with 1.0 mg/l IAA, 0.5 mg/l BAP, 0.3 mg/l GA₃, 0.8% agar, pH 5.8) for 5-7 weeks to induce shoots.

Shoots, detached from the callus, were grown on BGS medium (3-5 weeks) and then rooted by transfer to MS medium (with 0.5 mg/l NAA above) (3-5 days) followed by MS medium lacking hormones.

**RESULTS AND DISCUSSION**

The results are summarised in Table 1.

Division of Dimorphotheca protoplasts was initiated after 2-3 days and had reached the 8-celled colony stage by day 12. Protoplasts from ray florets failed to divide and division was delayed by 2 days for the other systems when grown in the dark.

<table>
<thead>
<tr>
<th>Species</th>
<th>Yield (x 10^6 g f.wt.)</th>
<th>Viability (%)</th>
<th>Source</th>
<th>MSP19M</th>
<th>K8P</th>
<th>K8P</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Dimorphotheca aurantiaca</em> (Giant Orange)</td>
<td>4.1</td>
<td>88</td>
<td>Leaf</td>
<td>4.1*</td>
<td>3.7*</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>87</td>
<td>Callus</td>
<td>29.0*</td>
<td>nt</td>
<td>80.1</td>
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<tr>
<td>(Glistening White)</td>
<td>6.6</td>
<td>81</td>
<td>Leaf</td>
<td>4.0*</td>
<td>2.9*</td>
<td>28.9</td>
</tr>
<tr>
<td>(’New Hybrids’)</td>
<td>4.9</td>
<td>86</td>
<td>Leaf</td>
<td>3.8*</td>
<td>2.5*</td>
<td>30.4</td>
</tr>
<tr>
<td><em>Rudbeckia hirta</em> (Marmalade)</td>
<td>2.3</td>
<td>74</td>
<td>Leaf</td>
<td>1.9*</td>
<td>1.3*</td>
<td>12.6</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>84</td>
<td>Callus</td>
<td>0</td>
<td>0</td>
<td>2.0</td>
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<tr>
<td></td>
<td>2.5</td>
<td>67</td>
<td>Cotyledon</td>
<td>2.7*</td>
<td>1.9*</td>
<td>18.7</td>
</tr>
<tr>
<td><em>R. lacinata</em> (Irish Eyes)</td>
<td>1.8</td>
<td>77</td>
<td>Leaf</td>
<td>2.1*</td>
<td>1.3*</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>90</td>
<td>Callus</td>
<td>0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>73</td>
<td>Cotyledon</td>
<td>1.9*</td>
<td>2.6*</td>
<td>18.5</td>
</tr>
<tr>
<td><em>R. purpurea</em> (Brilliant star)</td>
<td>1.3</td>
<td>89</td>
<td>Callus</td>
<td>nt</td>
<td>nt</td>
<td>8.5*</td>
</tr>
<tr>
<td></td>
<td>1.3</td>
<td>81</td>
<td>Floret</td>
<td>nt</td>
<td>nt</td>
<td>0</td>
</tr>
</tbody>
</table>

L, culture in light; D, dark conditions; nt: not tested. Best results were generally obtained for agarose solidified media except as shown* (liquid media). All experiments were replicated three times.