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Research note

Influence of stock plant pretreatment on gynogenic embryo induction from flower buds of onion

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Abstract

The gynogenic response of a range of onion genotypes to flower bud culture was compared using a two-step culture system. Embryogenic cultures and plantlets were produced from unpollinated ovules in whole flower bud explants 6 to 19 weeks after culture initiation. Preconditioning stock plants significantly influenced gynogenic embryogenesis. A ten-fold increase in embryogenesis was obtained when flower buds were cultured from stock plants maintained at 15 °C compared to 10 °C or the ambient temperature conditions of a glasshouse (maximum-minimum of 25–12.7 °C). A total of 49 embryos was obtained from 2660 cultured flower buds and 45% of plantlets were successfully acclimatised to glasshouse conditions. The majority of acclimatised plantlets were haploid (68%) but spontaneous double haploid plants (23%) were obtained from three genotypes.

The use of *in vitro* techniques to accelerate production of homozygous lines in onion, through induction of haploid plants and subsequent doubling of chromosomes, is of interest for the commercial production of F1 hybrid varieties and for the production of experimental lines for genetic studies. Gynogenic regeneration in onion has previously been reported (Muren, 1989; Keller, 1990; Campion and Alloni, 1990; Smith et al., 1991; Campion et al., 1992; Bohanec et al., 1995a); however, embryo induction rates are typically low, 0–17% (Campion and Alloni, 1990; Bohanec et al., 1995a; Geoffriau et al., 1997), which is a limiting factor for application of the technology in plant breeding. Overcoming low induction frequencies in gynogenic embryogenesis in onion have included studies of whole flower buds, ovaries and ovules as explants (Campion et al., 1992; Bohanec et al., 1995a), as well as medium composition (Bohanec et al., 1995b; Jakše et al., 1996).

Improved responses have been favoured by high sucrose concentrations (Muren, 1989) and supplements of 2,4-D (2,4-dichlorophenoxyacetic acid) and BA (6-benzylaminopurine) (Campion et al., 1992; Bohanec et al., 1995a, b; Jakše et al., 1996). In sugarbeet, cold pre-treatment of stock plants is reported to promote the frequency of regeneration from ovule cultures (Svirshchevskaya and Bromotov, 1994). The effect of raising stock plants under different environmental conditions on gynogenic embryogenesis has not been previously reported for onion. We show that pretreating the stock plant can significantly affect the frequency of gynogenic embryogenesis from whole flower bud cultures in a range of onion genotypes.

Bulbs of eight onion genotypes ('Balstora', 'Rocky', 'Sturon', 'AC 93032', 'AC 93030', 'Rocodoro', 'D165' and 'RCS 6151') including open-pollinated and hybrid varieties and breeding lines were field-raised in 1995 using standard horticultural practice. Bulbs were stored over winter and planted between January and April 1996, in 15 cm pots containing Levington's C2 compost with a surface layer of grit. Plants were initially raised under glasshouse conditions with min 18°C and natural daylight extended by artificial lighting to give a 14 h day. At the onset of bolting plants were either moved to growth chambers (10 or 15 °C) with natural daylight or were left

Table 1. Composition of culture media used

	M1	F6	A2	B1	C1
Macro and micro elements					
B5 (Gamborg, 1968)	+	–		–	–
MS (Murashige and Skoog, 1962)	–	–	+	+	+
BDS (Dunstan and Short, 1977)	–	+	–	–	–
Supplements (mg l^{-1})					
$NaH_2PO_4.H_2O$			250	250	170
Vitamins (mg l^{-1})					
m-Inositol	100	100	500	500	500
Thiamine-HCl	10	2	2	2	2
Pyridoxine-HCl	1	1	1	1	1
Nicotinic acid	1	1	1	1	1
Ca panthothenate	–	1	1	1	1
L-proline	–	–	200	200	–
Folic acid	–	–	1	1	1
Biotin	–	–	0.01	0.01	0.01
Glycine	–	–	0.2	0.2	0.2
Adenine sulphate	–	–	300	–	–
Growth regulators (mg l^{-1})					
TIBA	–	–	0.2	–	–
2iP	–	–	–	2	2
BA	2	2	–	–	–
IAA	–	–	–	–	1.5
NAA	–	–	–	1	–
2,4-D	2	2	–	–	–
Carbohydrate and gelling agent (g l^{-1})					
Sucrose	100	100	30	100	30
Agar	6	6	6	6	6

Media formulations are as described by Muren (1989) (M1), Campion and Alloni (1990) (A2, B1 & C1) and Campion et al. (1992) (F6).

to flower under glasshouse conditions. Plants maintained at 15 °C flowered under these conditions, while those maintained at 10 °C were then moved to 15 °C conditions when their umbels were about to open.

Flower buds with the pedicel attached were excised from umbels prior to anthesis and surface-sterilised in 1.7% (w/v) sodium dichloroisocyanurate for 6 min, followed by two rinses in sterile distilled water. The flower pedicel was then excised and 10 flower buds plated onto petri dishes containing 6 ml of induction medium, i.e. M1, F6 or A2 (see Table 1). Petri dishes were sealed with Nescofilm and incubated at 27 °C with a 16-h photoperiod provided by warm white fluorescent tubes with an irradiance of 35 μmol m^{-2} s^{-1}. After 20 days, flower buds were transferred to B1 media (see Table 1) and returned to the same culture conditions until embryos emerged.

Elongated regenerating embryos free of the ovary were transferred to C1 propagation media (see Table 1) in 100 ml glass jars and incubated at 22 °C with a 16-h photoperiod provided by Gro-lux fluorescent tubes until plantlets had developed. Basal swelling of the shoot leads to the formation of a small bulb on C1 media. Plantlets with well developed shoots and roots were potted in 2.5 cm modules containing a mix (1:1) of Levington's F2 compost and vermiculite. Plantlets were acclimatised for two weeks in propagators under the same culture conditions. Acclimatised plantlets were then transferred to 15 cm pots containing Levington's C2 compost and transferred to glasshouse conditions with min 18 °C and natural daylight. Ploidy levels of acclimatized plants were determined by flow cytometry analysis using material harvested from the youngest leaves of glasshouse-grown plants. Extraction of nuclei and flow cytometry analysis were performed by Plant Cytometry Services (PO Box 299, NL-5481 JG Schijndel, the Netherlands).

Culture initiations were performed on 18 occasions between July and August. On each occasion a minimum of 30 buds were excised from pre-treated stock plants of those genotypes with flower buds nearing anthesis, with 10 buds placed onto a petri dish of each induction medium. The availability of flower buds determined the number of buds cultured on each occasion and resulted in an unbalanced experimental design. Gynogenesis from flower buds excised from stock plants maintained in each of the three different raising conditions was compared. The number of flower buds producing regenerating embryos was recorded and the data analysed using a generalised linear model. Using a Poisson error and log link function, predicted values were generated from the model to exclude missing treatment combinations and allow comparisons to be drawn (McCullagh and Nelder, 1989).

A total of 960 and 1130 flower buds from all onion genotypes tested were cultured from pre-treated stock plants held at 10 and 15 °C respectively. From glasshouse-maintained stock plants, 570 flower buds were cultured but no flower buds from stock plants of 'Rocodoro', 'Sturon' and 'AC93032' were suitable for culture initiation. Pretreating stock plants influenced the responses obtained from cultured flower buds. Embryo induction was significantly improved (χ^2(df)=28.2(2), $p<0.001$) when flower buds were excised from stock plants maintained at a constant 15 °C. Flower buds excised from stock plants maintained at 15 °C were ten times more responsive than

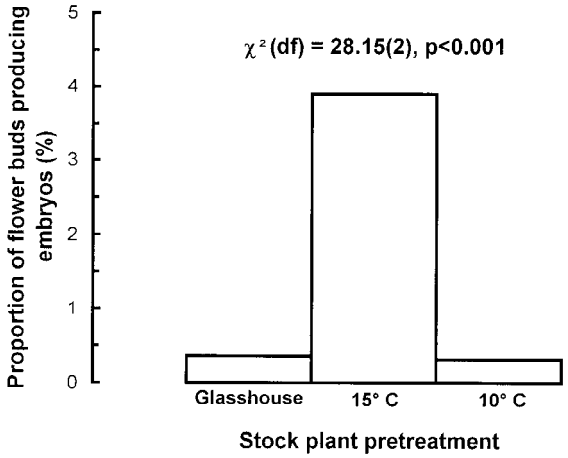

Figure 1. Influence of stock plant pretreatment on the frequency of gynogenic embryogenesis from cultured flower buds of onion. Significant differences were determined using a glm.

those taken from plants raised either under glasshouse conditions or held at 10 °C prior to flowering (Figure 1). The overall frequency with which flower buds of each genotype produced embryos varied significantly (χ^2(df)=41.6(7), $p<0.001$). No embryos were obtained from cultured flower buds of the Rijnsburger genotype 'Balstora'. The most responsive genotype was 'RCS 6151', an American hybrid, with 5.9% of cultured flower buds producing an embryo. Across all genotypes a significantly higher proportion of flower buds (2.9%) produced embryos when cultured on M1 induction medium (χ^2(df)=9.3(2), $p<0.05$) than on either A2 or F6 (1.5% and 1.1% respectively).

For most genotypes, embryos emerged 7–12 weeks after culture initiation, although hybrid genotype 'RCS 6151' embryos appeared between 6–19 weeks after culture initiation. Embryos emerged directly from the ovules; as they ruptured the ovary wall they were eventually pushed clear by the elongating root. Following transfer to propagation media developing embryos formed individual plantlets, and in some plantlets the basal portion of the shoot swelled to form a small bulb. Occasionally, proliferation of plantlets was observed. Over half of the embryos transferred from regeneration to propagation media did not survive. From a total of 2660 cultured flower buds, 49 embryos emerged (1.8%), of which 22 (0.8%) were successfully regenerated and acclimatised to glasshouse conditions. Flow cytometry analysis was performed on all acclimatised plantlets. The majority of acclimatised plantlets were haploid (68%) but spontaneous double haploid plants were also obtained (23%). During growth of plantlets under glasshouse conditions, plants produced flowers and seed was harvested from doubled haploids of one gynogenic line. Haploid plants tended to be less vigorous, reflected in reduced plant, umbel and flower bud size compared to doubled haploids.

The effect of genotype on the frequency of gynogenesis in onion has been reported previously (Smith et al., 1991; Campion et al., 1995; Jakše et al., 1995; Geoffriau et al., 1997). Similarly, induction media supplemented with 2,4-D and BA have generally been superior for gynogenic embryo induction (Bohanec et al., 1995a; Jakše et al., 1996). Cold pretreatments of stock plants of sugarbeet have improved gynogenic responses from cultured ovules. We found that responses of flower buds cultured from plants held initially at a constant 10 °C were equivalent to those from glasshouse raised stock plants (0.31 and 0.35% of flower buds responded respectively), but were significantly reduced compared to cultures from stock plants held at a constant 15 °C. Glasshouse stock plants experienced mean (±SE) weekly rises of 12.2 ± 0.51 °C from average minimum temperatures of 12.7 ± 0.28 °C up to average maximum temperatures of 25 ± 0.7 °C over the flowering period.

The principal benefits arising from the use of an optimised pretreatment for stock plants was the elimination of variation due to external factors which maximise gynogenic responsiveness in cultured flower buds of onion across the flowering season. Further experiments are required to determine whether the 15 °C pre-treatment effect is temperature-related or a consequence of reducing the fluctuations in temperature experienced by stock plants held under glasshouse conditions.

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