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A micropropagation technique for cauliflower (*Brassica oleracea var. botrytis*) to facilitate agrobacterium transformation

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Summary

A highly productive micropropagation technique was developed to facilitate Agrobacterium transformation in the recalcitrant brassica species cauliflower. Meristematic clusters were homogenised using a commercial blender and then graded through precision sieves (212–300) µm. From one curd was over 5000 explants were cultured in S23 liquid media supplemented with 0.2 mg L^{-1} of kinetin and 0.1 mg L^{-1} IBA and produced microshoots within 2 weeks. Several explant intensities (23, 34, 74 and 240 µL) per container containing 20 mL of culture media were investigated in terms of their effects on the subsequent growth ability of the microshoots. It was observed that the use of 34 µL per container gave the optimal result. Five blending durations were investigated (15, 30, 60, 90 and 120 s) . It was observed that the use of 30 s gave the optimal result. The explants were cultured in agitated liquid medium which optimized the production of thousands of plants from one mother curd. Twelve combinations of growth regulator plant growth regulators (NAA and Kinetin) were used to optimize shoot and root production. This protocol has large scale propagation potential of high quality progagules which will facilitate co-culture with Agrobacterium.

Key words: Cauliflower, micropropagation, propagules

Introduction

The main purpose of this study was to establish a protocol for cauliflower transformation using *Agrobacterium tumefaciens*. Several aspects of this research were investigated independently and they include the preparation of explants for cauliflower transformation.

Meristems are the most genetically stable part of a plant and in consequence the most suitable for production of true-to–type propagules or clones. Micropropagation using curd meristems has been used to maintain cauliflower parent lines (Crisp & Walkey, 1974) and for the early screening of curd quality (Crisp & Gray, 1979). Also this tissue has been used as a source of protoplasts (Yang *et al*., 1994) and artificial seeds of cauliflower plants (Kieffer, 1995). The technique of curd meristem micropropagation for cauliflower clonal propagation is now well established and Kieffer *et al*. (1995, 2001) have described a technique for rapid mass production of cauliflower propagules involving the homogenisation of curd tissues and they termed the protocol "microshoots". The microshoot technique produces tens of thousands of meristematic pieces in liquid culture in relatively small volumes (e.g. 1–2000 propagules in 20 mL) and the technique offers unique and exciting possibilities for mutagenesis and genetic transformation by facilitating rapid screening of

numerous propagules in culture. It is important to initiate growth of the explants to provide basic nutrients within the medium, this usually consist of a mixture of salts which provide the essential macro and micro elements as well as a carbon source (usually sucrose). The most widely used of the formulations available is that of Murashige & Skoog (MS) (Jansen *et al*., 1992).

Material and Methods

Plant material

Several varieties of F1 hybrid winter cauliflower (1. Clemen February heading, 2. Dionis January heading, 3. Redoutable February heading, 4. Mascaret March heading) were grown in the field in a raised bed at the University of Plymouth, Devon, UK according to good commercial practice and the curd harvested and stored at 4ºC until used. *In-vitro* micro shoots were produced in liquid according to the microshoot technique described by Kieffer *et al*. (1995, 2001).

Procedure

Large pieces of curd (1–5 cm) were surface sterilised firstly by immersing for 15 min 10% unthickened commercial bleach (sodium hypochlorite, 0.06% active chlorine), followed by three washings in sterile distilled water. Following surface sterilisation, explants were produced manually. The first step eliminated the mass of non-responsive tissue (stem branches) by shaving off the upper meristematic layer using a scalpel under sterile conditions in a laminar flow hood and discarding the stem branch material. The second step was a mechanical partial homogenisation of the selected meristematic tissue using a commercial blender (Waring model 800) at approximately 1700 rev. min^{-1} for 30 s followed by the use of precision sieves (600, 300, 212 µm aperture size) to rank the explants into the size classes 212–300 µm and 300–600 µm. The explant culture density was controlled by using a constant volume per container containing 20 mL of culture medium. Eighty to 100 culture containers were used per experiment and incubated on a shaker (approx. 50 revs min^{-1}). Culture media were derived from Murashige & Skoog (1962), according to Anderson & Carstens (1977) and supplemented with Kinetin (0.2 mg L^{-1}) and IBA (indol-3-butyric acid (0.1 mg L^{-1}) for the shoot development medium, and IBA only (0.2 mg L^{-1}) and agar (7 g L^{-1}) for the rooting medium (Kieffer *et al*., 1995). Culturing was carried out in the laboratory at 23ºC with adjacent lighting provided by white fluorescent tubes with a photoperiod of 16 h. The overall culture method was perfected by three practice attempts to help reduce contamination rate. The addition of antibiotic Plant Preservation Mixture (PPM) (1.0 mg L^{-1}) was routine to further reduce contamination risk (Fuller & Pizzey, 2001). All media, sieves and the Waring blender were sterilised by autoclaving at 121ºC for 15 m.

After 3 weeks growth, the number of growing propagules in each culture vessel with shoots and roots and bracts (green leafy fragments) was counted. The fresh weight of the total explants per container was also determined. Samples measurement were the number of explants in each pot and the weight of explants per pot. Twelve Plant Growth Regulator (PGR) combinations of Naphthalene acetic acid (NAA) and Kinetin were applied in tissue culture media Each culture pot contained 20 mL media.

Results

A. Effect of different blending durations on explants of size 212 & 300 µm

During explant development in agitated liquid medium, the majority of the good quality microshoots were floating at the surface of the culture medium, this was especially obvious when cultured at 23°C.

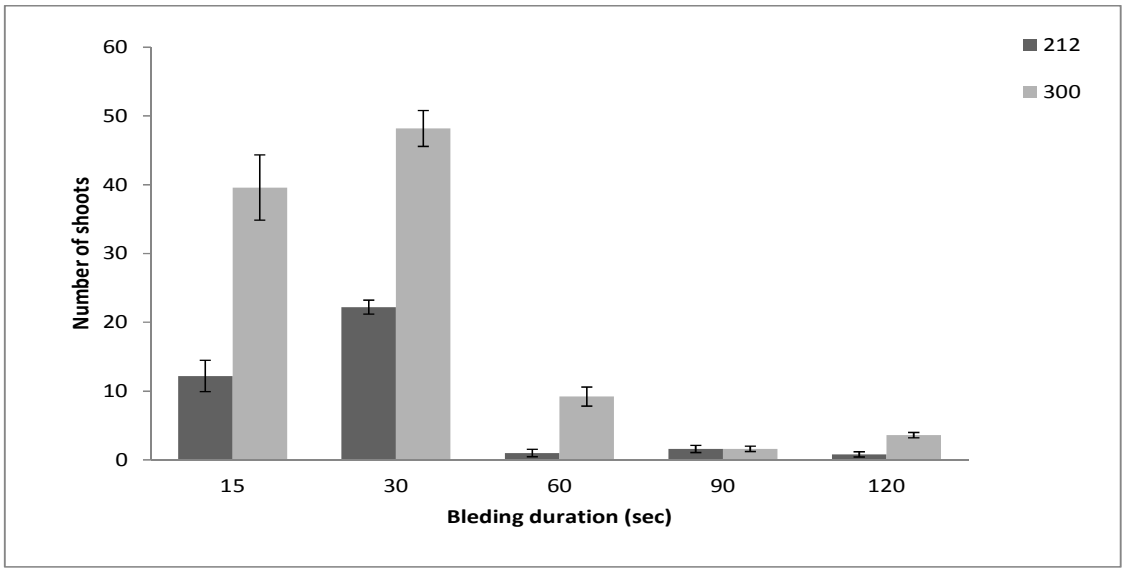
For the two size-classes the number of responding microshoots in culture for a constant volume of cultured micro explants decreased with blending duration as also found by Kieffer (1995).

The optimal treatment duration to obtain the maximum number of responding microexplants was 30s for both size-classes. The number of well-developed microexplants per container varied between 5–20 ± 10 for the 212–300 µm to 40 ± 20 for 300–600 µm size class (Fig. 1*a*).

Furthermore the weight of the responding microexplants in culture for a constant volume of cultured micro explants decreased with blending duration for both size-classes. The optimal treatment duration to obtain the maximum of responding microexplant weight was 30 s for both size-classes. The weight of well-developed microexplants per container varied between 40 g ± 10 g for the 212–300 µm and 80 g ±10 g for 300–600 µm size class (Fig. 1*b*).

The standard culture medium (supplemented with 0.2 mgL^{-1} Kinetin and 0.1 mg L^{-1} IBA) enabled regeneration of microshoots which often displayed early signs of polarisation with root hair like structures close to the root apex . The use of the same basal culture medium supplemented with 0.5 mg L^{-1} Kinetin and 0.1 mg L^{-1} NAA enabled direct regeneration of rooted microshoots. These micropropagules could be easily used in transformation experiments by co-cultivation with a bacterial strain (28ºC on a shaker) for at least 24 h.

A.



B.

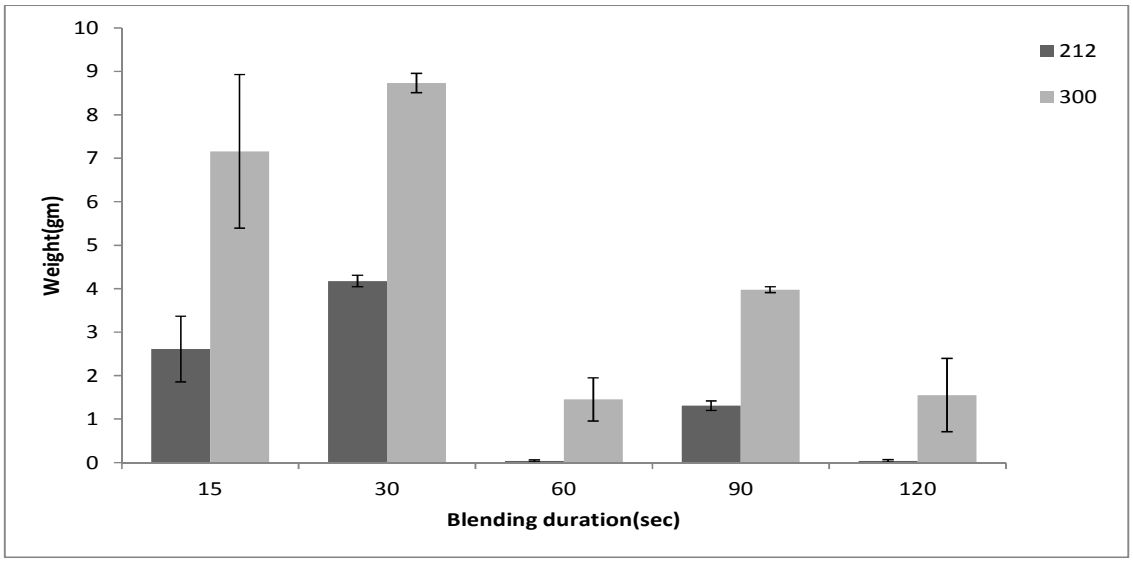


Fig. 1. Effect of blending duration on A. shoot number and B. weight.

Table 3. *The effect of plant growth regulators and time on the root length of microshoots (mm)*

Treatments	15 day	20 day	25 day	30 day	Mean
T1	0.40	0.00	9.00	4.00	3.35
T2	0.00	0.00	28.00	33.00	15.25
T3	0.20	0.00	20.00	39.00	14.80
T4	0.00	0.40	9.40	20.20	7.50
T5	0.00	0.00	1.00	0.00	0.25
T6	0.00	5.00	31.40	7.00	10.85
T7	0.00	3.00	25.00	15.40	10.85
T8	0.00	14.00	22.00	14.00	12.50
T9	0.00	0.00	0.00	0.00	0.00
T10	0.00	20.00	33.00	26.00	19.75
T11	1.20	34.00	42.00	29.00	26.55
T12	0.00	19.00	12.60	17.40	12.25
Mean	0.15	7.95	19.45	17.08	

Table 4. *The effect of PGR's and time on weight (g)*

Treats.	15 day	20 day	25 day	30 day	Mean
T1	0.001	0.001	0.009	0.015	0.007
T2	0.001	0.008	0.008	0.067	0.021
T3	0.003	0.007	0.031	0.061	0.026
T4	0.003	0.002	0.014	0.035	0.014
T5	0.052	0.114	0.071	0.126	0.091
T6	0.017	0.090	0.102	0.080	0.072
T7	0.016	0.042	0.061	0.069	0.047
T8	0.011	0.083	0.147	0.060	0.075
T9	0.015	0.085	0.078	0.046	0.056
T10	0.009	0.049	0.053	0.030	0.035
T11	0.003	0.088	0.166	0.121	0.095
T12	0.014	0.055	0.092	0.081	0.061
Mean	0.012	0.052	0.069	0.066	

Table 5. *ANOVA results* P *and LSD values of the source of variance*

	Shoot		Root		Weight	
Source of variance		LSD		LSD		
	P value	$_{(0.05)}$	*P* value	$_{(0.05)}$	*P* value	$LSD_{(0.05)}$
Treatment	0.000	4.394***	0.000	7.732***	0.000	0.034***
Days	0.000	2.537***	0.000	4.464***	0.000	0.020***
Treatment*Days	0.000	8.788***	0.000	15.464***	0.203	$0.069^{n.s}$

***-Highly significant, n.s-non-significant.

There were highly significant differences between PGR treatment, days and the interaction between them for shoot weight. The greatest weight was found in T11 (0.166 g), T8 (0.147 g), T5

(0.126 g), T11 (0.121 g), T5 (0.114 g) and T6 (0.102 g), and these treatments clearly differed from the rest (Table 4). These treatments can be used for the next experiment.

Discussion

The protocol reported enables the production of thousands of cauliflower propagules from one curd. The protocol relies on the use of pre-existing meristems, which probably accounts for the high genetic conformity observed within clones since meristems are genetically stable. Furthermore, the exceptional small size of the explants used should have the advantage of improving the efficiency of removal of virus infection already successful when bigger explants are used (Walkey, 1974).

The main objectives of the work described in this study was to develop an efficient protocol for genetic modification of cauliflower from curd. Development of an efficient gene transfer system largely depends on a rapid and reliable *in vitro* regeneration system for the desired plant species (Christy & Sinclair, 1992). This requires the establishment of an efficient regeneration system for cauliflower so that a sufficient number of shoots could be produced to allow the necessary level of replication in the regeneration and transformation experiments.

Optimization protocols for shoot regeneration

Shoot regeneration from these different tissues are affected by several variable factors. These variables include the type and character of explants and the medium composition (Gregco *et al*., 1984; Finer, 1987; Burrus *et al*., 1991; Sun *et al*., 1998; Muller *et al*., 2001; Dhaka & Kothari, 2002; Lingling *et al*., 2002; Flavio & Beatriz, 2005).

Effect of growth regulator on shoot regeneration

Different durations of blending were tested in this experiment (15, 30, 45, 60, 90 and 120 s). When the time of duration was increased the number of meristems destroyed was increased and 30 s duration gave least damage to the meristems. Secondly different sizes of sieve were used (212, 300 and 600 µm). The optimum size of sieve was obtained from 212 µm which gave uniform production of shoots in size and shape but less number of meristematic layer while the size class 300 µm gave huge number of shoots but included multiple shoots. Thirdly, different explant culture volumes were used (23, 34, 74 and 240 µL) in each pot, this meant when the volume increased the number of shoots per pot increased and *vice versa*.

The optimum duration was 30 s to obtain uniform shoots in shape and size less bracts less damage of meristem and highest growth capacity.

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