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Short communication

# Regeneration from leaf explants and protoplasts of *Brassica oleracea* var. *botrytis* (cauliflower)

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ABSTRACT

Adventitious shoot regeneration and protoplast isolation and culture were examined from leaf explants of *in vitro* shoot cultures of several cauliflower (*Brassica oleracea* var. *botrytis*) cultivars, sourced from Europe and Australia, was investigated with the aim to develop improved nuclear and plastid transformation protocols for this vegetable crop. Eight out of 10 cultivars regenerated shoots from at least 79% of leaf explants. Mesophyll protoplasts from leaves gave high yields and division frequencies. Growth of shoot cultures in large glass vessels with vented lids was the key factor in obtaining high protoplast division frequencies of up to 71% and at least 70% of protoplast calluses regenerating shoots.


## 1. Introduction

There is an extensive plant tissue culture literature on *Brassica* species (reviewed in Cardoza and Stewart, 2004), including research into the production of intra- and intergeneric hybrids of brassicaceae via protoplast fusion (Navrátilová, 2004). Shoot regeneration from cauliflower tissue culture has been reported by somatic embryogenesis (Deane et al., 1997; Leroy et al., 2000), but protocols aimed at *Agrobacterium*-mediated transformation rely on adventitious shoot formation from seedling explants. Cauliflower has been transformed via *Agrobacterium tumefaciens* (e.g. Bhalla and Smith, 1998), *Agrobacterium rhizogenes* (David and Tempé, 1988) and through direct DNA uptake into protocols from hypocotyls (e.g. Mukhopadhyay et al., 1991; Xue et al., 1997) or mesophyll cells (Radchuk et al., 2002; Nugent et al., 2006). Apart from transient expression data in broccoli (Puddephat et al., 1999) there are no reports of nuclear transformants of *Brassica oleracea* via *biolistics*. Interestingly, biolistics has been used to produce plastid transformants of *Brassica napus* (Hou et al., 2003) and *B. oleracea* (Liu et al., 2007) from seedling and leaf explants, respectively.

In this paper we report adventitious shoot regeneration from leaf explants and mesophyll protoplasts from cauliflower cultivars sourced from Europe and Australia. We found an increased shoot regeneration frequency from leaf explants and protoplast cultures of cauliflower, which should be useful when applied to *Agrobacterium*-mediated and direct DNA uptake transformation methods previously reported for cauliflower, but biolistics-mediated nuclear and plastid transformation of vegetable brassicas might also be achievable via leaf explants.

## 2. Materials and methods

### 2.1. Plant material

Seeds of European sourced cultivars of cauliflower (Thalassa, Arbon, Martian, Nautilus and Liberty) were obtained from Goldcrop Ltd. (Dublin, Ireland). Australian sourced cauliflower cultivars were obtained from Clause Tezier Australia, Melbourne, Australia (Thalassa), Yates (Quick Heart, All Year Hybrid, and Phenomenal Early) or Fairbanks Selected Seed Co. Pty. Ltd. (Melbourne, Australia) (Brittany and White Star). Seeds of the Indian cultivar 'Early Kunwari' were kindly provided by Prof. Pental, TATA Research Institute, India. Seeds were stored at 4 °C prior to use.

### 2.2. Explant preparation

Cauliflower *in vitro* shoot cultures were established from seedlings as described previously (Nugent et al., 2006). All plant growth regulators (Duchefa) and silver nitrate ($AgNO_3$) were filter sterilised (Sartorius, 0.22 μm) and added to media after autoclav-

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*Abbreviations:* BAP, 6-benzyladenine; MS, Murashige and Skoog medium; NAA, α-naphthalene acetic acid.

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ing. Leaf explants were cut from leaves with a scalpel blade, at approximately 1 $cm^2$ sections, avoiding the mid-vein, with a cut edge on each side, and cultured abaxial side down and in sterile disposable Petri dishes (90 mm × 15 mm) containing 20 ml of MS (Murashige and Skoog, 1962) based shoot induction medium (SIM) (45 μM BAP/5.4 μM NAA/29.4 μM $AgNO_3$) (after Dunwell, 1981), at 22 °C under a 16 h photoperiod provided by cool, white fluorescent lights at photon flux density of 50 μmol/($m^2$ s). The plates were sealed with surgical tape (Leukopore). The number of adventitious shoots/roots for all explants was counted after a total period of 8 weeks in culture. An additional treatment with Thalassa leaf explants tested the effect of bombardment using a gene gun on regeneration frequency. Leaves were bombarded with gold particles (0.6 μm diameter) coated with plasmid DNA (pZB1, Nugent et al., 2006) using a PDS 1000/He Biolistic gene gun (BioRad). A rupture disc pressure of 1100 psi, partial vacuum pressure between 28 in Hg and a target distance of 6 cm was used for bombardment.

### 2.3. Protoplast isolation and culture

Mesophyll protoplasts were isolated from leaves of shoot cultures grown as described above and cultured according to a published protocol (Nugent et al., 2006) as modified from Pelletier et al. (1983). Shoot cultures were grown in several types of culture vessels to determine the effect on protoplast yield and division frequency. Shoot cultures established from 10-day-old germinated seedlings were transferred to either Magenta vessels GA-7 (Sigma, V8505), Phytacon Vessels (Sigma, P-5557), Glass jars (145 mm × 85 mm, Phytotechnology Labs, C956) or Glass jars with vented lids. The vented lids of glass jars were prepared by cutting a (3 cm diameter) hole in the middle portion of the lids using a scalpel

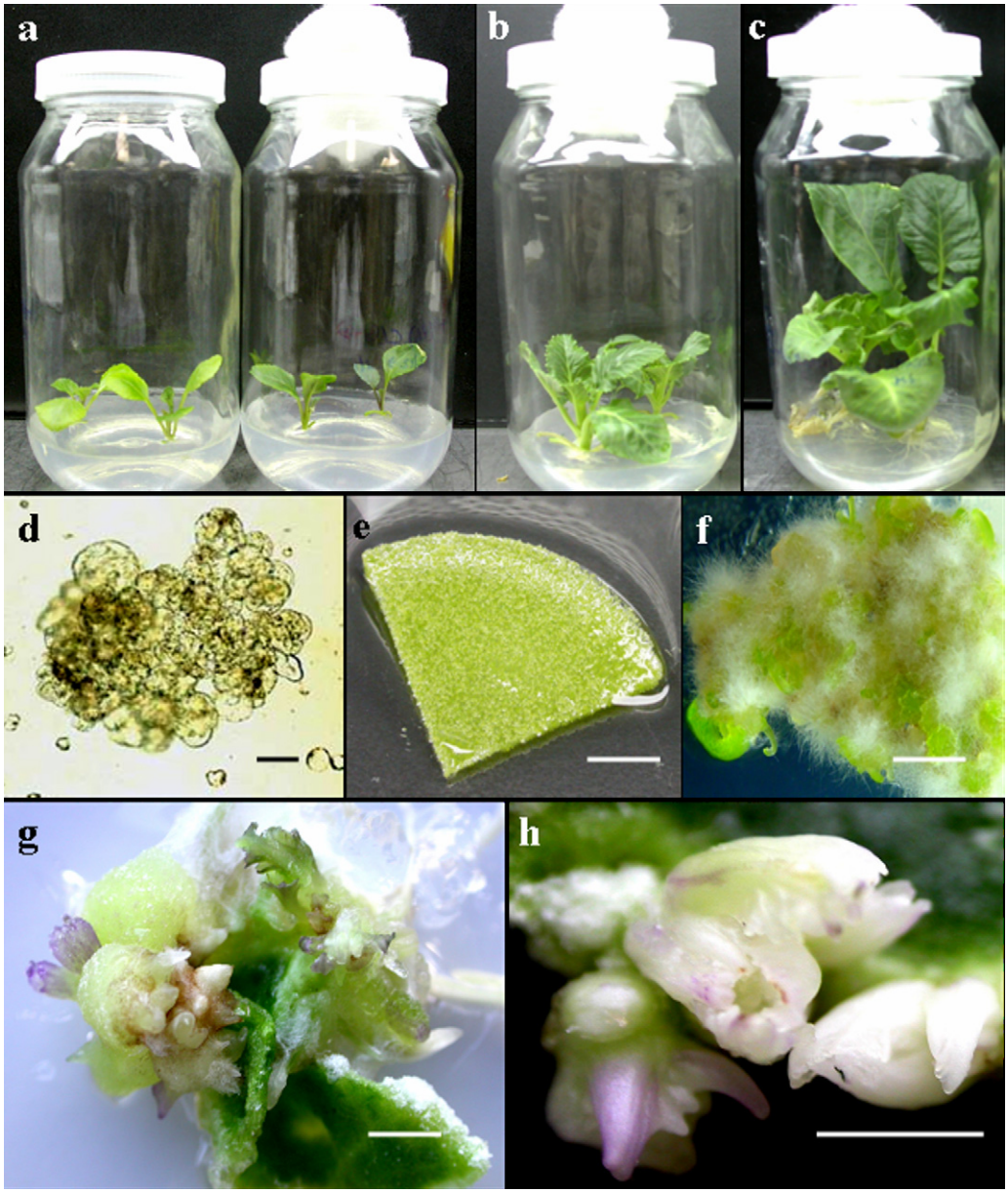


**Fig. 1.** Cauliflower *in vitro* shoot cultures as a source of mesophyll protoplasts and shoot regeneration from leaf explants. (a) Early establishment of shoot cultures after 2 weeks in vented (left) and unvented glass jars (right); (b) vigorous growth of shoot cultures after 4 weeks in vented glass jars; (c) shoot culture growth at the end of 8 weeks; (d) protoplast microcolony in agarose at 20 d from protoplast isolation (bar = 50 μm); (e) protoplast colonies in agarose at 30 d, medium D; (f) shoot and root regeneration from protoplast calli after 40 d (medium E); (g and h) adventitious shoot bud differentiation from leaf explants after 2 weeks on SIM; bar = 1 mm.

blade, and plugging the hole with absorbent cotton wool. All the culture vessels were used with identical medium (MS + 30 g/l sucrose +4 g/l Phytagel). These shoot cultures were maintained at 25 °C under 16 h light/8 h dark light conditions. Protoplast isolation experiments were carried out three times and each time new shoot cultures were taken from germinated seedlings and inoculated in the different containers. Protoplast viability was measured with fluorescein diacetate (Widholm, 1972).

### 2.4. Data analysis

The number of explants that showed callus formation, root formation and shoot formation per explant were counted. Percentage of explants forming callus, root and shoot response, and also root and shoot number from each explant, was recorded. Normality test, data transformation, ANOVA and Tukey's tests were performed on root and shoot percentage response and their number for the significant differences at $p < 0.05$ among the explants and cultivars using MINITAB 14 software. Data were log transformed to improve the normal distribution where necessary. For the protoplast experimental data, yield, viability, division and shoot regeneration from calli were also analysed with MINITAB using the same methods.

## 3. Results and discussion

### 3.1. Shoot regeneration from leaf explants of cauliflower cultivars

Leaf explants from shoot cultures were examined as alternative explants to seedling tissue for shoot regeneration for transformation studies. Adventitious shoot buds were visible from cut leaf margins, callus and across the leaf surface from 2 weeks on SIM. Buds may be initially unpigmented, pigmented or green (Fig. 1g and h). There was no significant difference between the mean number of shoots regenerated amongst cultivars within each cultivar group (Table 1). The Australian sourced cultivars were more uniform in response, with Early Glory and Quick Heart being the choice cultivars, given their significantly higher regeneration frequencies. Thalassa was a superior cultivar amongst the European sourced cultivars, but did not have a significantly higher frequency than the others. Leaves from shoot cultures maintained for longer than three subculture periods of 3–4 weeks showed a reduced capacity for shoot regeneration, but explants from leaves bombarded with gold particles coated in plasmid DNA with the BioRad gene gun did not (Table 1). Several of the tested cultivars have demonstrated the highest regeneration frequency from leaves of any cauliflower cultivar yet reported. Seedling hypocotyls are preferred for regeneration and transformation of brassicas (Puddephat et al., 1996; Cardoza and Stewart, 2004), however, there are few reports of shoot regeneration from *B. oleracea* leaves. Regeneration frequencies from *B. oleracea* leaves have been reported as 0 (Bhalla and de Weerd, 1999), 19% (Cheng et al., 2001), 31% (Ovesná et al., 1993), 50% (Dunwell, 1981) and 79% (Cao and Earle, 2003) but there have been few reports of shoot number per explant. Mature leaves regenerated fewer shoots than younger leaves of rapid cycling *B. oleracea* and broccoli (Cheng et al., 2001; Cao and Earle, 2003), but the number of shoots per explant and regeneration frequency was much lower than found in cauliflower in our study.

Adventitious shoots arose from cut edges of leaves in our study, but also from across the leaf lamina and from around vascular tissue (data not shown). However Bhalla and de Weerd (1999) reported no shoots were regenerated from cauliflower leaf explants which did not contain leaf veins, but shoots regenerated at low frequency from leaf vein explants without leaf lamina tissue attached. It remains to be shown whether these shoots develop directly or indirectly from mesophyll cells or vascular parenchyma in cauliflower, given that several studies have shown that brassica adventitious shoots arise from vascular parenchyma cells in seedling and leaf explants (e.g. Hachey et al., 1991; Mukhopadhyay et al., 1992; Sharma et al., 1993; Akasaka-Kennedy et al., 2005). This is interesting as plastid transformants of *B. oleracea* have been regenerated from leaf explants after biolistics delivery of plasmid DNA (Liu et al., 2007). There is the possibility therefore that these plastid transformants regenerated from the plastid rich mesophyll cells, given that vascular parenchyma cells are a smaller target for biolistics-delivered DNA and occur deeper in the leaf tissue than many of the mesophyll cells. Nonetheless, the utility of leaves for generating nuclear transformants of brassica directly via *A. tumefaciens* or biolisitcs has yet to be reported. Leaves have been used for *A. rhizogenes*-mediated transformation of brassicas (e.g. Christey et al., 1997), but transgenic plants were generated indirectly from hairy-root cultures. It does show that *Agrobacterium* may transform some brassica leaf cells, but it has not been demonstrated that *Agrobacterium* can transform leaf cells capable of forming shoot primordial or somatic embryos.

**Table 1**
Adventitious shoot regeneration from *in vitro* leaves of European (bold) and Australian sourced cultivars of cauliflower

| Cultivar | Number of explants | Root regeneration (%) | Number of roots, mean ± S.D.[a] | Shoot regeneration (%) | Number of shoots, mean ± S.D.[a] |
|---|---|---|---|---|---|
| Liberty | 120 | 85 | 5.3 ± 4.5ab | 60 | 3.6 ± 4.7a |
| Nautilus | 120 | 88 | 5.9 ± 4.8ab | 72 | 4.1 ± 4.1a |
| Early Kunwari[b] | 120 | 96 | 6.3 ± 3.1a | 88 | 4.1 ± 3.5a |
| Thalassa | 120 | 84 | 4.2 ± 3.0b | 82 | 5.6 ± 4.8a |
| Thalassa 3rd[c] | 120 | 88 | 4.3 ± 2.8b | 53 | 2.6 ± 3.1a |
| Thalassa (unshot)[a] | 120 | 98 | 9.0 ± 5.0a | 85.8 | 7.3 ± 6.5a |
| Thalassa (shot)[d] | 120 | 99 | 7.5 ± 3.9a | 85 | 5.4 ± 4.6a |
| Early Glory | 150 | 59.3B | 2.8 ± 2.5A | 92A | 7.3 ± 3.2A |
| Phenomenal Early | 150 | 61.3B | 2.7 ± 2.4A | 80B | 5.5 ± 3.2AB |
| All Year Hybrid | 150 | 59.3B | 2.8 ± 2.6A | 80B | 5.2 ± 3.0B |
| Quick Heart | 150 | 60B | 2.6 ± 2.4A | 82.6AB | 6.1 ± 3.2AB |
| White Star | 150 | 70.6AB | 3.2 ± 2.6A | 80B | 5.5 ± 3.3AB |
| Brittany | 150 | 70.6AB | 3.2 ± 2.4A | 78.6B | 5.4 ± 3.2AB |

Shoot induction medium (SIM): MS/45 μM BAP/5.4 μM NAA/29.4 μM $AgNO_3$. Explant responses recorded at 5 weeks, recorded as percentages and mean number ± standard deviation (S.D.). Numbers followed by a different letter are significantly different after Tukey's test ($p < 0.05$).

[a] Unshot control.

[b] Cultivar sourced from India.

[c] Leaf explants sourced from shoot cultures on their third subculture cycle. All other leaves taken from shoot cultures after one subculture.

[d] Leaf explants shot with DuPont PDS/1000He gene gun, with plasmid pZB1 (Nugent et al., 2006).

**Table 2**
Protoplast responses from shoot cultures grown in various vessel types (cv. Brittany)

| Cultivar | Protoplast yield ($\times 10^6$ #pp/g fwt) | Protoplast viability (%) | Protoplast division (10 d) (%) | Shoot regeneration from calli ($n = 200$) (%) |
|---|---|---|---|---|
| Magenta | 1.07a | 77.6a | 11.8a | 32a |
| Sigma | 1.6a | 77a | 10.9a | 31a |
| Glass jar | 2.0a | 87b | 41.6b | 57b |
| Glass jar with vented lid | 2.1a | 96.6c | 70.1c | 78c |

Yield is measured as the number of protoplasts per gram of fresh weight of leaf tissue. Protoplast viability measured with FDA (Widholm, 1972). Division recorded at 10 d after isolation. Numbers followed by a different letter are significantly different ($p < 0.05$) after Tukey's test.

**Table 3**
*In vitro* responses of protoplasts isolated from various Australian sourced cauliflower cultivars

| Cultivar | Protoplast yield ($\times 10^6$ #pp/g fwt) | Viability (%) | Protoplast division (10 d) (%) | Calli regeneration ($n = 200$) (%) |
|---|---|---|---|---|
| Thalassa | 2.6a | 96.0a | 71.7a | 76.3a |
| White Star | 2.2a | 97.0a | 69.3a | 71.3a |
| Brittany | 2.1a | 96.7a | 69.9a | 71.7a |
| Quick Heart | 2.0a | 96.0a | 53.3c | 67.3a |
| All Year Hybrid | 2.6a | 96.2a | 67.7a | 68.3a |
| Phenomenal Early | 2.7a | 96.7a | 52.0c | 64.7b |
| Early Glory | 2.5a | 97.5a | 60.3b | 67.7a |

Yield is measured as the number of protoplasts per gram of fresh weight of leaf tissue. Protoplast viability measured with FDA (Widholm, 1972). Division recorded at 10 d after isolation. Shoot regeneration from calli recorded 5 weeks after transfer to medium F. Numbers followed by a different superscript letter are significantly different ($p < 0.05$) after Tukey's test.

### 3.2. Protoplast isolation and culture

Regeneration from cauliflower mesophyll protoplasts has been more successful with protocols based on Pelletier et al. (1983) than Glimelius (1984) (reviewed in Kik and Zaal, 1993). As such, efficient protoplast isolation and culture, incorporating such factors as agarose embedding, were established for cv. Thalassa (data not shown), based on a protocol developed at Rijk Zwaan BV, Netherlands, adapted from Pelletier et al. (1983) (see Nugent et al., 2006). Shoot cultures of Brittany were then used in the container experiment where similar yields of mesophyll protoplasts were obtained from leaves from the four container types (Table 2). Significantly higher protoplast viability, division and shoot regeneration was obtained from shoot cultures grown in glass jars (Table 2). Protoplasts from leaves of shoot cultures grown in large, vented culture vessels produced significantly higher division frequency than those from leaves from unvented containers of the same or smaller size (Fig. 1a). Shoot cultures developed vigorously in vented jars (Fig. 1b and c). The importance of ventilation of culture vessels and Petri dishes has also been demonstrated in other brassica tissue culture systems. Micropropagated cauliflower seedlings grew better *in vitro* in vented containers with or without added $AgNO_3$ (Zobayed et al., 1999) and a significant improvement in culture of *B. napus* protoplasts was obtained when shoot cultures, used as a source of mesophyll protoplasts, were grown on a modified basal medium in large, vented glass jars (Dovzhenko, 2001). However this medium made cauliflower shoots chlorotic in our study (data not shown), but large culture vessels was a key improvement for cauliflower (Table 2).

No significant difference in protoplast yield or viability was found among the seven Australian sourced cultivars. In all of these cultivars, high yields, viabilities and division frequencies were obtained (Table 3). Protoplast microcolonies were well developed by 20 d of culture (Fig. 1d) and masses of green colonies easily visible by eye in agarose discs by 30 d on medium D (Fig. 1e) and early stages of shoot and root regeneration were visible on medium E by 40 d after protoplast isolation (Fig. 1f). Only Phenomenal Early showed significantly lower shoot regeneration from protoplast calli than the other cultivars. Protoplast division and regeneration in our study were the highest yet reported for cauliflower (e.g. Kik and Zaal, 1993) and comparable to the broccoli cultivar Green Comet (Robertson and Earle, 1986). With a division frequency reliably around 70% for several cultivars including Thalassa, using the modified protocol in combination with vented vessels, we have a sound basis for improved transgenesis from protoplasts of cauliflower, compared to earlier studies (Radchuk et al., 2002; Nugent et al., 2006).

In conclusion, this study has found that regeneration from several of the European and Australian sourced cultivars exhibit the most efficient regeneration from leaf explants and mesophyll protoplasts reported to date for this species. The utility of this high regeneration frequency in transformation systems is under examination.

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