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Regeneration of Pea (*Pisum sativum* L.) by a cyclic organogenic system

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Abstract In a five-step procedure, plants were regenerated from meristematic tissue initiated from nodal tissue in four pea cultivars ('Espace', 'Classic', 'Solara', and 'Puget'). In step 1, stem tissue with one node (1-cm size) was subcultured on medium containing thidiazuron. As a result multiple shoots were produced, appearing normal or swollen at their bases. The multiple shoots were subcultured in the same medium, resulting in the formation of a green hyperhydric tissue in the swollen bases of the multiple shoots, which is fully covered with small buds [bud-containing tissue (BCT)]. In step 2, BCT fragments were isolated and subcultured in the same medium and, as a result, they were able to reproduce themselves in a cyclic fashion. In step 3, subculture of BCT on medium supplemented with a combination of gibberelic acid, 6-benzyladenine and α-naphthalene acetic acid (NAA), resulted in the formation of shoots, which were rooted in step 4 on medium supplemented with 0.5 mg/l NAA, indole-3-acetic acid (IAA) or indole-3-butyric acid. In step 5, in vitro plants were transferred to the greenhouse for acclimatisation and further development. The four varieties tested were all able to produce meristematic tissue, suggesting that its production is genotype independent.

Keywords Thidiazuron · Cyclic · Shoot regeneration · Adventitious regeneration · Meristems

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Introduction

Pea (*Pisum sativum* L.) is an important crop in Northern Europe. It is grown for its seeds, which are considered as a high quality and relatively cheap source of protein, used for cattle feed and in the human diet. Resistance to viruses, lowering of anti-nutritional factors, and improving protein composition and quality, are important goals in pea breeding. Improvement of these goals can probably only be accomplished with genetic modification, since peas' natural variation is limited (Christou 1997 and references therein). Pea, like most legume species is recalcitrant to genetic modification, although protocols for genetic modification of pea have been described (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). In the procedures described by Schroeder et al. (1993) and Polowick et al. (2000), segments of embryogenic axis are used. Bean et al. (1997) and Nadolska-Orczyk and Orczyk (2000) use cotyledonary nodes as starting material. After infection with *Agrobacterium tumefaciens*, multiple shoots are formed, some of which contain genetically modified shoots. However, these systems have drawbacks such as a low efficiency (about 2–5% of the initial seeds), high frequency of escapes and the occurrence of chimeric genetically modified plants (Bean et al. 1997; Grant et al. 2003). These disadvantages might be related to the fact that the majority of the shoots are regenerated from existing meristems, and might be reduced significantly if plants were regenerated from newly formed meristems (adventitious regeneration). Adventitious regeneration can be obtained either by somatic embryogenesis or by shoot organogenesis, and both types of regeneration can be either direct or indirect via a callus phase. The latter is preferred over direct regeneration for genetic modification as the callus phase is ideal for selection of genetically modified cells and, subsequently, plants. However, callus-based regeneration systems have the disadvantage that they have a much higher chance of yielding plants with somaclonal variation than direct regeneration.

Somatic embryogenesis has been described in pea (Loiseau et al. 1998; Griga 1998, 2002 and references therein). In our own laboratory it was found that the described protocols are genotype-dependent, somatic embryos are regenerated directly without callus phase, and the efficiency is low (unpublished results). The multicellular origin of somatic embryos in pea (Loiseau et al. 1998; Griga et al. 2002) might be another constraint in employing this embryogenic system for genetic modification, as it might result in chimeric genetically modified somatic embryos, as was observed in cassava (Raemakers et al. 2001) and walnut (Escobar et al. 2000). Shoot organogenesis has been reported in pea using hypocotyl explants (Ochatt et al. 2000) and immature cotyledon explants (Grant et al. 1995). The protocol described for hypocotyls has a low regeneration frequency and is genotype-dependent (Ochatt et al. 2000) and has not been combined with genetic modification. The protocol described for cotyledons has been combined successfully with genetic modification. As in the other systems above described, multiple shoots are formed. Also in this adventitious system, the efficiency is low, there is a large variation between experiments, and often a high frequency of escapes (Grant et al. 1998). In this report, we present a novel regeneration system with both adventitious and non-adventitious properties. An organogenic/meristematic tissue is formed from nodal tissue, which can be maintained in a cyclic fashion, giving it callus-like properties. Furthermore, we describe the regeneration of fertile plants, which were grown successfully in the greenhouse.

Materials and methods

General

A step-by-step approach was used to test a range of pea (*P. sativum* L.) commercial varieties ('Espace', 'Classic', 'Solara', and 'Puget') with the aim of developing a novel regeneration protocol. Each experiment consisted of three replicates with 18–20 explants per replicate.

Media and growth conditions

Unless stated otherwise, basic medium contained Murashige and Skoog (1962) salts and vitamins, 30 g/l sucrose and 7.5 g/l micro agar (MS3). The pH was adjusted to 5.7 prior to autoclaving (121°C for 15 min). All growth regulators were added filter-sterilised to the medium after autoclaving. Media were dispensed into 9-cm Petri dishes (25 ml) or into 9 cm jars (310 ml). All cultures were incubated in a growth chamber at a temperature of 24°C, a photoperiod of 16 h and an irradiance of 40 μmol m^{-2} s^{-1}.

Preparation of plant material

Seeds from commercially important pea varieties ('Espace', green pea semi-leafless; 'Classic', yellow pea semi-leafless; 'Solara', green pea semi-leafless, kindly provided by Cebeco Zaden (Lelystad, The Netherlands), and 'Puget', green pea, semi-leafless, kindly provided by S. Bean, John Innes Centre, Norwich, UK), were surface-sterilised in 70% ethanol for 1 min, subsequently immersed in 1.6% sodium hypochloride for 20 min, and rinsed three times with sterile distilled water. The seeds were then cultured for 36 h in jars containing 50 ml water (10 seeds per jar) after which they were cultured on MS3 for further growth.

Step 1: Induction of bud-containing tissue on one-node stem explants

Shoots of in vitro plants were cut into one-node pieces of about 1 cm. Cuttings were placed on MS3 medium or MS3 medium supplemented with 1.1 mg/l or 2.2 mg/l thidiazuron (TDZ) or 0.55 mg/l or 1.1 mg/l 6-benzyladenine (BAP). Every 4 weeks, the newly formed shoots were subjected to the same procedure of cutting one-node stem pieces and subculture on the fresh medium. A vitrified callus-like bud containing tissue (BCT), covered with buds, was formed near the bases of the shoots.

Step 2: Isolation and multiplication of BCT

Isolated fragments (2–5 mm) of BCT were placed on MS3 medium or MS3 medium supplemented with 0.5 mg/l, 1.1 mg/l, 2.2 mg/l, 4.4 mg/l, or 8.8 mg/l TDZ or 0.25 mg/l, 0.55 mg/l, 1.1 mg/l, 2.2 mg/l, or 4.4 mg/l BAP. Every 4 weeks the developed BCT was subdivided and subcultured on the same medium.

Step 3: Regeneration of shoots from BCT

BCT multiplied for 6 months on MS3 supplemented with 2.2 mg/l TDZ was used as explant material for regeneration of plants. BCT cultured for 4 weeks on MS3 supplemented with 2.2 mg/l TDZ, was cultured on medium supplemented with B_5 salts and vitamins (Gamborg et al. 1968), 30 g/l sucrose, 7.5 g/l micro agar ($B_5$3) and all possible combinations of gibberellic acid (GA_3), α-naphthalene acetic acid (NAA) and BAP, at a concentration of 1 mg/l for 5 weeks. After 3 weeks the explants were transferred to the same medium.

Step 4: In vitro rooting of shoots derived from BCT

Shoots developed from 6-month-old BCT cultured on $B_5$3 medium supplemented with 1 mg/l GA_3 were subcultured in jars on $B_5$3 medium supplemented 0.5 mg/l IAA, or 0.5 mg/l IBA or 0.5 mg/l NAA (Ochatt et al. 2000) for rooting. After 5 weeks the apical part (5 cm) of the shoots was subcultured on the same medium for an additional period of 5 weeks. After this period, rooted and non-rooted plants were transferred to the greenhouse for acclimatisation.

Step 5: Greenhouse rooting and acclimatisation of plants derived from BCT

In vitro plants, with or without roots, produced as described above, were planted in pots containing standard potting soil and maintained under constant temperature of 18°C and relative moisture of 100% for further development. Plants without roots were covered with plastic to reduce water evaporation prior to root formation.

Low temperature scanning electron microscopy

BCT samples cultured for 4 weeks on MS3 supplemented with 2.2 mg/l TDZ were placed on a specimen holder and immediately plunged into liquid N_2 (−196°C). The frozen samples were transferred to a cryo-transfer unit (CT 1500-HF Oxford Instruments, Oxford, UK). The unit consists of a cryo-preparation chamber under high vacuum (10^{-6} Pa) dedicated to a low temperature scanning electron microscope (LT-SEM; JEOL, model 6300 F,

Tokyo, Japan) and a cryo-stage inside the microscope. The specimens were placed inside the cryo-chamber at −85°C, and freeze-dried for 2 min at −85°C and 10^{-7} Torr. The samples were then sputter-coated with 10 nm platinum. LT-SEM was used to examine the coated callus-like tissue at 5–10 kV, keeping the temperature of the specimens at −180°C.

Ploidy level analysis

To identify the ploidy level of the shoots produced, flow cytometry was performed by Plant Cytometry Services (Schijndel, The Netherlands).

Results

Induction of BCT on stem node explants

BCT was first observed in experiments where stem pieces were cultured on MS3 medium supplemented with 2.2 mg/l TDZ to maximise multiple shoot formation. BCT is characterised as a vitrified, dark green, callus-like tissue, round in shape, covered with buds, and was formed in the swollen parts of the multiple shoots.

One-node explants of four commercial pea varieties were subcultured for three consecutive cycles on media with various amounts of cytokinins to optimise BCT initiation. The results obtained with cultivars Puget and Solara are shown in Table 1. Explants cultured on MS3 medium grew normally and formed rooted plants with one shoot per explant in the first cycle. Similarly, in the two subsequent subculture cycles, only plants with a single shoot were formed. However, only 20% of these plants formed roots compared to 100% in the first cycle. The same response was observed for the other two varieties.

Addition of BAP to MS3 medium resulted in the formation of a single shoot without roots per cultured node stem explant in the first cycle. Similar results were obtained in the two subsequent subculture cycles. Multiple shoot formation was never observed in any of the four cultured varieties. In variety Solara, the addition of 1.1 mg/l BAP induced in vitro flower formation in 25% of the cultured node explants. However, in vitro seed formation was not observed.

Addition of TDZ to MS3 medium resulted in the formation of multiple shoots (3–4 shoots per node explant) in the first cycle, of which 30% had swollen bases. In the second cycle all subcultured node stem explants formed multiple shoots, all swollen at their bases. Some explants formed BCT on the swollen bases of the multiple shoots. In Puget, BCT was produced in 4% of node explants on 1.1 mg/l TDZ and in 15% of node explants cultured on 2.2 mg/l TDZ. This increased in the third cycle to 10% and 85% of subcultured node stem explants cultured on 1.1 mg/l and 2.2 mg/l TDZ, respectively. In Espace and Classic, the frequencies were comparable to those of So-lara and Puget (Table 1) in all cycles.

Isolation and multiplication of BCT

Preliminary results showed that BCT was not able to develop into plants while attached to the bases of the multiple shoots. On MS3 medium supplemented with 2.2 mg/l TDZ, the amount of BCT increased with time and the multiple shoots grew slowly. On MS3 medium supplemented with mixtures of BA, NAA and GA_3, the BCT died and the multiple shoots grew further.

In the next set of experiments, BCT was isolated from the bases of the multiple shoots and pieces of 2–5 mm were cultured on MS3 medium supplemented with cytokinins (BAP or TDZ). The general response of BCT was equal for all four genotypes. Table 2 shows the effects of cytokinins on BCT multiplication for the varieties Puget and Classic after 4 weeks of culture. On MS3 medium, around 40% of the explants of Puget and Classic survived and the rest of the explants turned brown within 2 weeks and died (Table 2). When MS3 medium was supplemented with 0.25–4.4 mg/l BAP, 38–50% of the explants from Classic survived, and for Puget this varied from 42% to 90%. Upon addition of 0.5–8.8 mg/l TDZ to MS3 medium, all explants survived and browning was not observed for Puget. For Classic all explants survived on MS3 medium supplemented with 4.4 mg/l or 8.8 mg/l TDZ, while at lower concentrations 47–72% of the explants survived.

Surviving BCT pieces cultured on MS3 medium became light green, lost their hyperhydric nature, and formed one or two short and thick shoots. On MS3 medium supplemented with BAP, most explants formed only one or two short and thick shoots and, at a low frequency, short and thick shoots together with BCT (Table 2). Addition of BAP did not result in formation of pure BCT. When TDZ was supplemented to the MS3 medium, either thick short shoots were formed or new BCT was initiated, or a mixture of both. Lower concentrations of TDZ stimulated shoot formation whereas higher concentrations of TDZ stimulated BCT formation. On MS3 medium supplemented with 2.2 mg/l or more TDZ, 50–63% of the BCT pieces were reproduced in pure state (Table 2) in the variety Puget.

In this way, BCT was maintained for more than 2 years by subculturing pieces of 2–5 mm every 4 weeks on MS3 supplemented with 2.2 mg/l TDZ. When larger pieces of 10–20 mm were used, BCT had a stronger tendency to form shoots instead of pure BCT and hence smaller pieces were preferred for routine purification and maintenance of BCT.

LT-SEM was performed to illustrate the nature of BCT. Figure 1b,c shows that bud-like structures, which appeared on the surface of BCT, are bud or shoot initials. After 4 weeks of culture on MS3 medium supplemented with 2.2 mg/l TDZ, BCT was fully covered with meristems at a density of 1 per mm^2. Some buds contained leaf primordia and shoot meristems, whereas others were at earlier stages (Fig. 1c).

Table 1 Effects of three cycles of subculture of one-node stem explants on medium supplemented with 6-benzyladenine (BAP) and thidiazuron (TDZ) in the pea (*Pisum sativum* L.) varieties Puget and Solara. Values are the mean of three replicates with 50–60 explants in total. *SSR* Single shoots with roots, *SS* single shoots without roots, *MS* multiple shoots without roots, *BCT* bud-containing callus tissue on the bases of the multiple shoots, *Pug* cv. Puget, *Sol* cv. Solara

Growth regulators (mg/l)	Response in first cycle (%)								Response in second cycle (%)								Response in third cycle (%)							
	SSR		SS		MS		BCT		SSR		SS		MS		BCT		SSR		SS		MS		BCT	
	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol	Pug	Sol
TDZ																								
0	100	100	0	0	0	0	0	0	20	20	80	80	0	0	0	0	20	20	80	80	0	0	0	0
1.1	0	0	0	0	100	100	0	0	0	0	0	0	96	95	4	5	0	0	0	0	90	85	10	15
2.2	0	0	0	0	100	100	0	0	0	0	0	0	85	86	15	14	0	0	0	0	15	5	85	95
BAP																								
0.55	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0
1.1	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0	0	0	100	100	0	0	0	0

Plant regeneration from BCT

BCT cultured for 4 weeks on MS3 medium supplemented with 2.2 mg/l TDZ was subcultured on $B_5$3 medium supplemented with various growth regulators for regeneration of shoots. In all four varieties tested, the response of BCT to the different media was similar. Table 3 shows the results for the varieties Puget and Espace after 5 weeks of culture. Depending on the growth regulator used, between 40% and 90% of the explants survived, and the rest became brown and died. A higher survival rate was observed when BAP and NAA, with or without GA_3, were used, and a lower rate was seen in the absence of growth regulators or when GA_3 with or without NAA or BAP was used.

Surviving explants formed shoots in all media tested after 5 weeks of culture. Two different types of shoot formation were observed depending on the presence or absence of GA_3 in the medium. Addition of GA_3 to $B_5$3 medium, alone or in combination with NAA or BAP, resulted in the formation of a high number of shoots (10–22), which were hyperhydric. The shoots completely covered the surface of BCT, giving it a bushy appearance (Fig. 2a). When these shoots were isolated from BCT and subcultured in the same medium, their hyperhydric appearance shifted to a normal (non-hyperhydric) one and new shoots were formed from the remaining BCT cells at the bases of the shoots. On the contrary, on $B_5$3 medium without GA_3, only 2–10 non-hyperhydric shoots developed per explant (Fig. 2b). These shoots developed normally when they were isolated and subcultured on the same medium and they did not form any new shoots from their bases. Shoots produced on media containing GA_3 were thinner, and generally longer, compared to shoots obtained on medium without GA_3.

In vitro rooting of shoots derived from BCT

Preliminary experiments showed that shoots (2–3 cm) derived from BCT cultured on medium supplemented with cytokinins alone or together with NAA or GA_3 did not form roots on $B_5$3 medium supplemented with auxins. Also, shoots isolated from 2-year-old BCT were not capable of forming roots in the various media tested (not shown).

In the results for the variety Puget presented in Table 4, shoots (2–3 cm in size) derived from 6-month-old BCT cultured for 5 weeks on medium supplemented with 1 mg/l GA_3 were used. Root formation was not observed in any treatment after 5 weeks of culture. The apical parts of shoots (5 cm) were subcultured in the same medium. In the second subculture, 50% of the shoots cultured in $B_5$3 medium supplemented with 0.5 mg/l IBA formed roots. When shoots were cultured in $B_5$3 medium supplemented with 0.5 mg/l NAA or 0.5 mg/l IAA, 24% and 9% of the plants rooted, respectively. Independent of the three auxins used, callus formation preceded root formation, but the roots formed were attached to the stem and functional.

Table 2 Effects of different concentrations of TDZ and BAP on proliferation of the BCT in pea (*P. sativum* L.) varieties Puget and Classic. Values are the mean of three replicates with 50–60 explants in total

Growth regulators (mg/l)	Explants surviving the treatment (%)		Explants producing only shoots[a] (%)		Explants producing a mixture of shoots[a] and BCT (%)		Explants producing only BCT (%)	
	Puget	Classic	Puget	Classic	Puget	Classic	Puget	Classic
TDZ								
0	40	43	100	100	0	0	0	0
0.5	100	47	50	63	50	37	0	0
1.1	100	55	50	48	50	52	0	0
2.2	100	72	8	9	42	91	50	0
4.4	100	100	8	8	42	47	50	45
8.8	100	100	2	4	35	48	63	48
BAP								
0.25	42	40	100	100	0	0	0	0
0.55	90	39	94	100	6	0	0	0
1.1	90	38	94	100	6	0	0	0
2.2	80	47	94	97	6	3	0	0
4.4	75	50	90	96	10	4	0	0

[a] Shoots were short and thick (as shown in Fig. 2b)

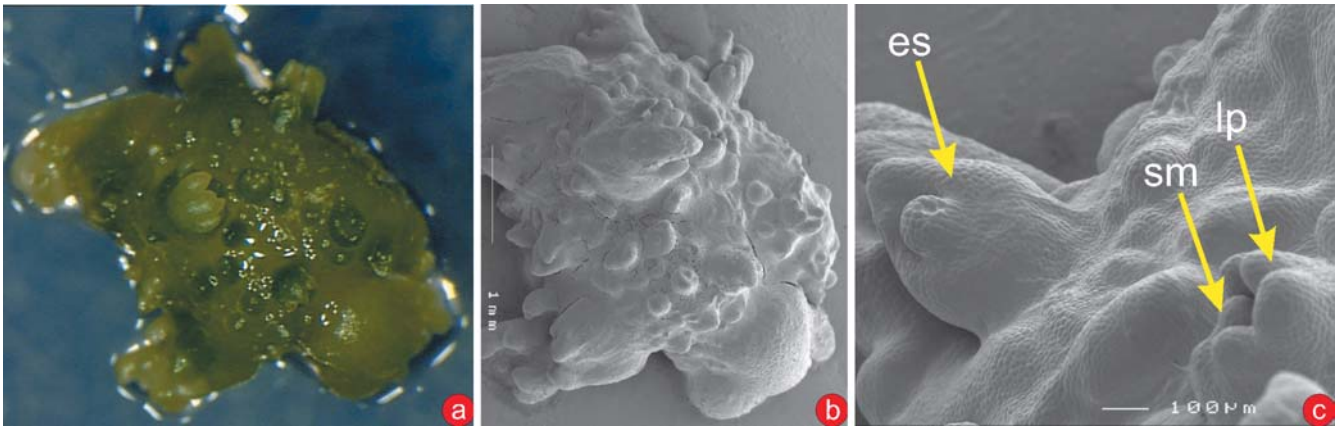

Fig. 1a–c Development of meristematic bud-containing tissue (BCT) after culture of one-node stem segments of pea on medium supplemented with 2.2 mg/l thidiazuron (TDZ). **a** Normal image of BCT. **b** Scanning electron micrograph of the same explant showing that globular structures on the callus surface are developing meristems. **c** Not all buds were in the same developmental stage: some formed leaf primordia (*lp*) and shoot meristems (*sm*), whereas others were in earlier stages (*es*)

Table 3 Effects of different growth regulators on shoot production of 4-week-old BCT in pea (*Pisum sativum* L.) varieties Puget and Espace (results after 5 weeks of cultivation). Values are the mean of three replicates with 50–60 explants in total. *NAA* α-naphthalene acetic acid, GA_3 gibberellic acid

Growth regulators (mg/l)			Explants surviving the treatment (%)		Explants forming long thin shoots (%)		Explants forming short thick shoots (%)		Number of shoots per responding explant	
GA_3	BAP	NAA	Puget	Espace	Puget	Espace	Puget	Espace	Puget	Espace
0	0	0	45	40	0	0	100	100	2[a]	3[a]
1	0	0	50	40	100	100	0	0	14[b]	12[b]
0	1	0	55	50	0	0	100	100	5[a]	5[a]
0	0	1	60	50	0	0	100	100	8[a]	6[a]
0	1	1	90	80	0	0	100	100	10[a]	8[a]
1	1	0	40	40	100	100	0	0	22[b]	20[b]
1	0	1	50	45	100	100	0	0	20[b]	19[b]
1	1	1	70	65	100	100	0	0	10[b]	10[b]

[a] Shoots were short and thick (as shown in Fig. 2b)
[b] Shoots were thin and long (as shown in Fig. 2a)

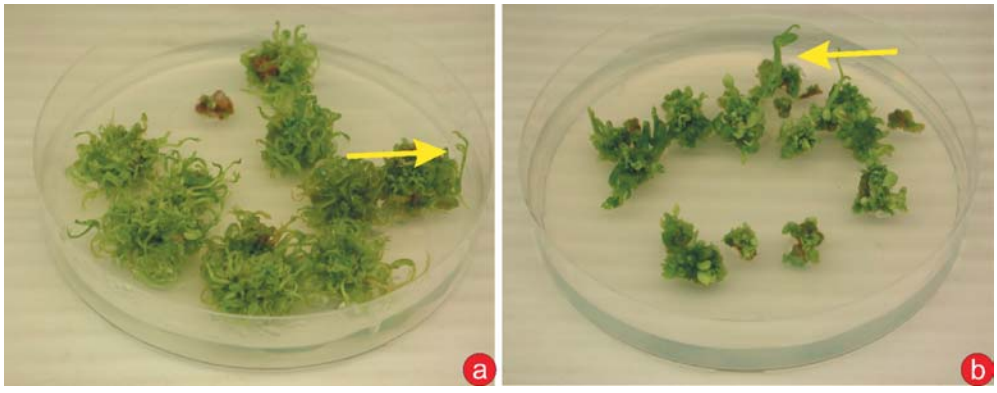

Fig. 2 Plant regeneration from BCT: shoots produced in the presence (**a**) or absence (**b**) of GA_3

Table 4 Root formation (>1 cm) of shoots obtained from 6-month-old BCT after two cycles of subculture on media supplemented with different auxins. Shoots were derived from media containing GA_3 (thin shoots) from the pea (*Pisum sativum* L.) variety Puget. Values are the mean of three replicates with 50–60 explants in total. *IBA* Indole-3-butyric acid

Auxin	Explants rooted in the first cycle of subculture (%)	Explants rooted in the second cycle of subculture (%)
0.5 mg/l IBA	0	50
0.5 mg/l IAA	0	9
0.5 mg/l NAA	0	24

When IAA was used, more vigorously growing plants developed than when IBA or NAA were used. Smaller plants were produced in the presence of NAA, which also had increased callus formation at their bases, compared with shoots rooted in the presence of IBA or IAA.

Acclimatisation of plants derived from BCT

All plants that rooted in vitro survived the transfer to the greenhouse. Within a period of 6–8 weeks, they flowered and subsequently formed fertile seeds. Of the plants that failed to form roots in vitro, 80% formed roots during acclimatisation and developed similarly as those that had rooted in vitro. Plants derived from BCT had the same ploidy level (2×), as control plants obtained from seeds.

Discussion

This study describes a novel regeneration protocol for pea (*P. sativum* L.). In a five-step procedure (Fig. 3), plants were regenerated from BCT—a meristematic/organogenic callus-like tissue. In step 1, BCT is initiated from one-node explants. In step 2, the BCT is isolated, purified and maintained. Regeneration of shoots from BCT and rooting are steps 3 and 4, respectively. Finally, the plants are transferred to the greenhouse in step 5. All the varieties tested were able to produce BCT and regenerated plants, showing that this new regeneration system is applicable to a broad range of varieties. TDZ was essential for the induction and maintenance of BCT. When the TDZ concentration was 2.2–8.8 mg/l, about half of the BCT explants initiated only new BCT. At a lower concentration of TDZ all BCT explants initiated a mixture of new BCT and shoots. Only a few shoots were obtained from the low TDZ treatment and these shoots did not form roots on auxin-supplemented medium. Therefore other media were tested for shoot development. The survival of BCT cultured on medium supplemented with GA_3 was low compared to medium supplemented with BAP and NAA. However, because the number of formed shoots and their ability to form roots was higher in GA_3-supplemented medium, that medium was preferred for shoot development.

It is well known that cytokinins such as TDZ suppress the growth of apical meristems and instead induce excess formation of lateral meristems, resulting in multiple shoots (Sanago et al. 1996). Popiers et al. (1997) reported the formation of hyperhydric tissue in pea together with the formation of multiple shoots after prolonged exposure to TDZ. In their system, only shoots isolated from multiple shoots developed into complete plants. Most probably, their medium for plant development was not optimal for formation of shoots from the hyperhydric tissue.

We observed that multiplication and maintenance of BCT on TDZ-supplemented medium for a period exceeding 2 years reduces the capacity of the regenerated shoots to form adventitious roots. Madsen et al. (1998) and Bean et al. (1997) also observed that long exposure of pea to either TDZ or BAP inhibited the capacity of shoots to form adventitious roots. Bean et al. (1997) used grafting techniques to transfer rooted plants to the greenhouse. Here it is shown that non-rooted in vitro plants form ex vitro roots. The plants grew normally in the greenhouse, looked phenotypically like seed-derived plants, did not have altered ploidy levels, and formed viable seeds. Plants derived from these seeds were also similar to wild-type plants. However, more research is needed to determine if plants derived from BCT that has been maintained for a

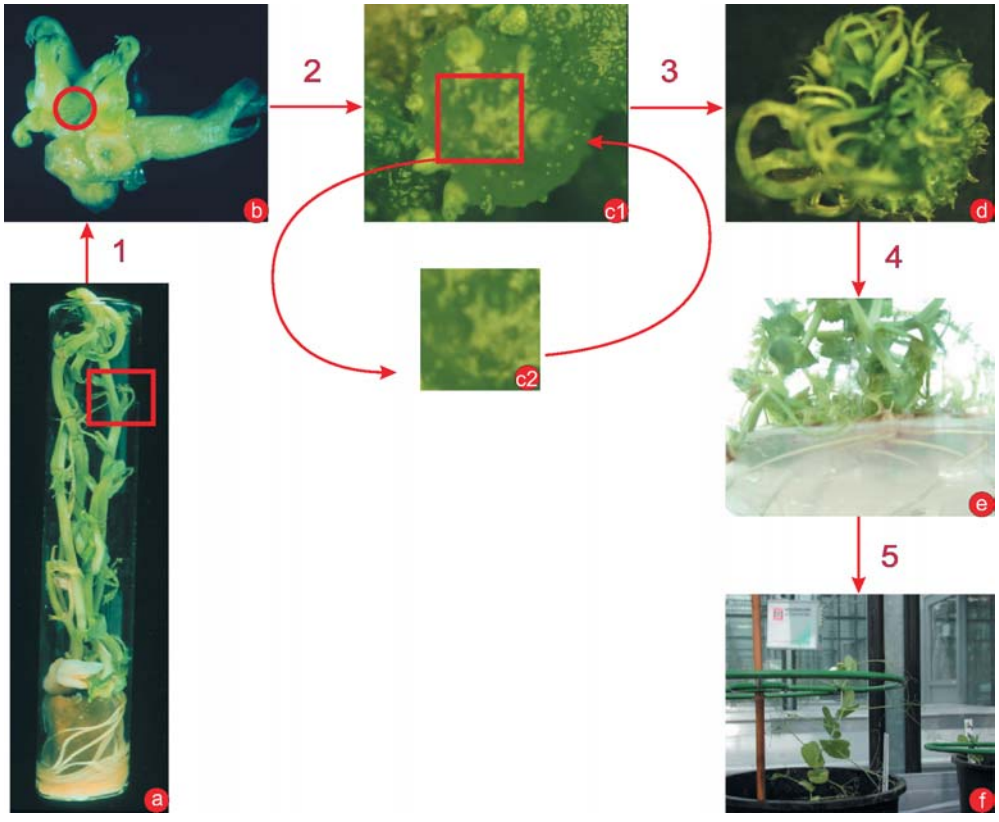

Fig. 3a–f A five-step procedure for the initiation of BCT and subsequent regeneration of plants in pea (*Pisum sativum* L.); steps *1–5* are indicated. **a** Pea plant obtained from seed with one-node explant (*box*), **b** production of swollen multiple shoots with meristematic BCT at their base (*circle*), **c**1 isolation and subculture of the BCT shown in **b** resulting in pure BCT, **c**2 suculturing BCT from **c**1 cut in fragments resulted in its reproduction in a cyclic manner, **d** formation of shoots from BCT, **e** rooting of shoots derived from BCT, **f** BCT-derived plant grown in the greenhouse (step 1: 8 weeks, step 2: 4 weeks, step 3: 5 weeks, step 4: 5 weeks (replication of this step optional since non-rooted in vitro plants can be transferred directly to the greenhouse), step 5: 6–8 weeks)

prolonged period of time are affected by somaclonal variation.

The regeneration protocol has characteristics both of an adventitious and a non-adventitious system. The ability of BCT to be maintained in a cyclic manner confers adventitious characteristics. However, the BCT itself is differentiated; it is covered with small meristems, which are most probably the source of the formation of new buds in the next multiplication cycle, giving it non-adventitious characteristics.

The ability of the BCT to reproduce itself might be beneficial for obtaining genetically modified plants. For successful genetic modification it is necessary that genetically modified cells have the ability to develop independently from wild type cells, and that the growth of genetically modified cells is favoured. This is usually accomplished by coupling the gene(s) of interest to genes governing resistance to antibiotics or herbicides. The appropriate antibiotic or herbicide is added to the medium and consequently only transgenic cells are able to divide. In most crops, selection is applied during a callus phase (undifferentiated cells). After the selection phase, the callus is cultured for regeneration. In pea, the regeneration systems that have so far been used for transformation are based on cytokinin-induced direct formation of shoots either from existing meristems or from newly formed meristems (Schroeder et al. 1993; Grant et al. 1995; Bean et al. 1997; Nadolska-Orczyk and Orczyk 2000; Polowick et al. 2000). The major drawbacks of these methods are low selection efficiency of the regenerating shoots and the often highly chimeric status of the transformed plants. Most probably this is caused by the fact that meristems are complex, multicellular structures in which it is difficult for individual cells to divide independently. The main difference between the regeneration system described here and the regeneration systems used by Schroeder et al. (1993), Grant et al. (1995), Bean et al. (1997), Nadolska-Orczyk and Orczyk (2000), and Polowick et al. (2000) to obtain genetically modified plants is the cyclic multiplication of BCT versus the linear development of shoots. In preliminary experiments, BCT has been subjected to transformation experiments using *A. tumefaciens* carrying the luciferase reporter gene. Luciferase-positive BCT was obtained and cultured repeatedly on TDZ-supplemented medium. During this process selection was based only upon luciferase activity. This resulted in complete luciferase-positive BCT and subsequently in complete luciferase-positive plants (data not shown), indicating the potential for genetic modification of the regeneration system described here.

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