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Establishment of embryogenic cell suspension cultures of garlic (*Allium sativum* L.), plant regeneration and biochemical analyses

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Abstract Embryogenic cell suspension cultures of garlic (*Allium sativum* L.) were initiated in liquid medium from friable embryogenic tissue. The optimal parameters for culture maintenance were: (1) an initial cell density of 1–4% (v/v); (2) medium renewal every 14 days and subculturing every 28 days; (3) a low 2,4-dichlorophenoxyacetic acid concentration (0.1–0.3 mg/l). Cultures regenerated during a 14-month period. The cell suspension cultures differentiated embryos following transfer to a semi-solid embryo induction medium, with histological studies confirming and characterising the embryogenic nature of the process. Forty percent of these embryos converted into plantlets, which produced micro bulbs in vitro. The composition of the sulphur compounds of the micro bulbs obtained from cell suspension embryo-derived plantlets differed slightly from those produced by in vitro shoot proliferation-derived plantlets, but after two cycles of multiplication in the field these differences had disappeared.

Keywords Somatic embryogenesis · Histology · Sulphur compounds

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Abbreviations

BAP	6-Benzylaminopurine
CS	Cell suspension-derived plantlet
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole-3-acetic acid
NAA	α-Naphthaleneacetic acid
SCV	Sedimented cell volume
SP	In vitro shoot proliferation-derived plantlet

Introduction

All commercial garlic (*Allium sativum* L.) cultivars are sterile (Etoh and Simon 2002; Kamenetsky and Rabinowitch 2001) and must be propagated vegetatively. Consequently, the chance of disease transmission is increased (Novak 1990), particularly as garlic varieties are standardly infected with various viruses (Dolores et al. 2002; Lot et al. 1994).

Methods for virus elimination and the production of virus-free material have been developed using meristem-tip culture (Ayabe 2001; Chovelon et al. 1990; Senula et al. 2000). Various researchers applying different micro-propagation protocols have obtained a limited multiplication rate with respect to the production of virus-free material (Barandiaran et al. 1999; Haque et al. 1997; Kahane et al. 1992; Kim et al. 2003; Myers and Simon 1998; Nagakubo et al. 1993; Robledo Paz et al. 2000; Zheng et al. 2003). As such, embryogenesis might be applicable in scaling-up the propagation of healthy garlic tissues (Xue et al. 1991); for example, a somatic embryogenesis process was reported recently (Fereol et al. 2002). Nevertheless, to date, the multiplication rates for producing virus-free garlic plants have been too low to satisfy the economic requirements for a practical mass propagation system. Plant production from embryogenic cell suspension culture-derived somatic embryos is a possible alternative. Due to the high medium-to-tissue contact in liquid culture system, media effects are rapid, and embryo development can be more strictly controlled than with a solid support system.

There have been limited reports on garlic suspension cultures. Nagasawa and Finer (1988) reported suspension cultures of callus proliferating as nodular clumps, but they did not obtain true cell suspensions or plant regeneration. Barrueto Cid et al. (1994) established garlic suspension cultures and regenerated plants, but they did not mention embryogenic characteristics nor the regeneration frequency. Moreover, their protocol required a high concentration of 2,4-D (1.1 mg/l), thereby risking somaclonal variation (Al Zahim et al. 1999).

The objective of the investigation reported here was to establish and characterise a reliable protocol for garlic proliferation via embryogenic cell suspension cultures. We investigated the effect of several parameters on cell multiplication and plant regeneration to determine and optimise conditions for maintaining the cultures. Special attention was directed towards keeping the 2,4-D concentration as low as possible. Histological studies ascertained the embryogenic capacity induced in cell suspension cultures. Chemical analysis was performed on micro bulbs from cell suspension embryo-derived plantlets (CS) and in vitro shoot proliferation-derived plantlets (SP) as a quality trait to distinguish between these two garlic proliferation strategies.

Materials and methods

Plant material

Garlic (*Allium sativum* L.) cultivar Morasol (INRA, France) was used in this investigation. This cultivar belongs to the Group-I temperate varieties according to the physiological classification of Messiaen et al. (1993). It was developed by INRA in 1994 through clonal selection and meristem-tip culture of the Spanish population Morado. Bulbs free of onion yell dwarf potyvirus (OYDV) and leek yellow stripe virus (LYSV) were used for initiating embryogenic cell suspension cultures. These bulbs were harvested for 4–5 months, stored at 15°C and then transferred for 3 weeks to 5°C before the experiments were initiated in order to break dormancy. The explants were young leaf sections from surface-sterilized cloves. These explants initiated embryogenic calluses as reported by Fereol et al. (2002). Shoot-tip proliferation was carried out as reported by Kahane et al. (1992).

Culture conditions

Initiation of the cell suspension cultures

The embryogenic calluses were sub-cultured repeatedly every 45 days on callus maintenance medium (CMM, Table 1). After 5 months of culture, they had formed a mixture of compact, semi-friable and friable calluses capable of regenerating somatic embryos. These calluses were used to initiate cell suspension cultures. Callus (0.25 g) was cultured in six 10-ml multi-well dishes containing 5 ml/well of liquid suspension medium (SM, Table 1) based on N6-modified salts (Chu et al. 1975). The cultures were incubated at 24–26°C in the dark with continuing agitation (100 rpm). After 14 days, 4 ml of the medium was removed and replaced by 4 ml of fresh medium. After 28 days post-culture initiation of the callus in the liquid medium, a cell suspension was initiated; it was filtered throw an 800-µm pore-sized sieve and the filtrate placed in a sterile graduated test tube. Following sedimentation of the filtrate, the sedimented cell volume (SCV) was measured.

Table 1 Solid and liquid media[a] used for garlic embryogenic cell suspension cultures and embryo regeneration

Nutrients (mg/l)	CMM	SM	EPM
Macro nutrients (modified)[b]	(B5)	(N6 m)	(N6 m)
KNO_3	2,500	2,850	2,850
$(NH_4)_2SO_4$	134	463	463
NaH_2PO_4, $2H_2O$	150	400	400
$CaCl_2$, $2H_2O$	150	440	440 q
$MgSO_4$, $7H_2O$	250	185	185
Micro nutrients[c]	(MS)	(H)	(H)
Vitamins	(B5)	(B5)	(B5)
Nicotinic acid	1	1	1
Pyridoxine	1	1	1
Thiamine HCl	10	10	10
Myo-inositol	100	100	100
Malt extract	100	100	100
Amino acid			
Proline	230		
Glutamine		150	150
Growth regulators			
2.4.D	0.5	0.3	0.1
IAA	0.2		
NAA	0.2		
kinetin	0.1		0.5
BAP		0.1	
Sucrose (g/l)	60	45	45
Agar, Phytagel	3g	0	3
p^H before autoclaving	5.8	5.8	5.8

[a]CMM, Callus maintenance medium; EPM, embryo production medium; SM, suspension medium

[b]N6 m, N6 modified salts (Chu et al. 1975); B5, Gamborg vitamins (Gamborg et al. 1968)

[c]H, Heller (Heller 1953); MS, Murashige and Skoog (Murashige and Skoog 1962)

Establishment of the cell suspension culture

The cell suspension cultures initiated above were sub-cultured in multi-well dishes by transferring 0.06–0.48 ml of SCV in 2-ml aliquots of the previous SM medium into a well to which 4 ml of fresh SM medium had been added to give an initial volume of 6 ml. The regeneration potential of the cell suspension cultures was studied by plating the former onto semi-solid embryo production medium (EPM, Table 1). The number of embryos was counted 2 months after plating.

Recordings

The following variables were measured: (v1) the SCV rate (final SCV/initial SCV), which is a measure of the growth of the cell suspension cultures after 14 days or 28 days post-subculture; (v2) the mean number of regenerated embryos per milliliter SCV plated on EPM medium; (v3) the total production of embryos potentially possible, taking in consideration the growth rate of the cell suspension culture during a 28-day subculture (v1), the regeneration potential per milliliter SCV plated (v2) and the quantity of initial SCV (q ml) in culture that we wish to consider—V3=v1×v2×q ml.

Experimental treatments

Several parameters were tested for their effect on maintaining the cell suspension cultures and on embryo production.

Initial cell densities The quantities of SCV cultured in 6-ml aliquots of SM medium were: 0.06, 0.12, 0.18, 0.24, 0.30 and 0.48 ml. These concentrations (ml/6 ml) were expressed as the respective percentages 1%, 2%, 3%, 4%, 5% or 8% (ml/100 ml). (v1) was measured at 14, 28, 42 and 56 days of culture, while (v2) was measured at 42 days of subculture, without medium renewal or subculturing.

Periodicity of medium renewal The periodicity of medium renewal was 7, 14, 21 or 28 days. The initial cell density was 4% (0.24 ml SCV/6 ml); (v1) was measured at 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of culture for each periodicity of medium renewal, while (v2) was measured at 42 days of culture. Subculture was not performed.

2,4-D concentration 2,4-D concentrations were 0.1, 0.3 or 0.5 mg/l. The initial cell density was 4% (0.24 ml SCV/6 ml); (v1) and (v2) were measured at 28 days over three subsequent cycles of subculture. The medium was renewed every 14 days, and sub-culture was performed every 28 days.

Age of the cell suspension cultures From 1 to 15 months post-culture initiation, (v1) and (v2) were measured at each subculture. Initial cell density was 4% (0.24 ml SCV/6 ml), with the medium being renewed every 14 days and subculture performed every 28 days. The cell suspension cultures were filtered through an 800-μm pore-sized sieve every 56 days and the filtrates were retrieved to continue experiments.

Comparison of sulphur compound composition The concentration of alliin (*S*-allyl-cysteine sulfoxide), GLUAlCS (γ-glutamyl-*S*-allyl cysteine) and GLUPeCS [γ-glutamyl-*S*-(*trans*-1)-propenyl- cysteine] was measured on (CS) and (SP) micro bulbs.

Statistical analysis

Our results were analysed using the linear model procedure of the SAS statistical package version 8.2 for windows (SAS Institute 1982). A one-way analysis was designed for variables v1, v2 and v3, including six replicates of one well for v1 and four or five replicates of one well for v2, v3. A replicate for v1 corresponded to the same as for v2, v3. The results analysed were means of treatments. Multiple comparisons of means were performed using Tukey's student range test at the 5% level of confidence.

Histological study

Both suspension cells and cells plated on semi-solid medium in petri dishes were collected at different stages, fixed, dehydrated and stained according to Fereol et al. (2002).

Sampling procedure for chemical analysis

Alliin, GLUAlCS and GLUPeCS were measured according to Arnault et al. (2003). One gram of the fresh bulb was homogenised with 3 ml methanol/water (80/20, v/v) + 0.05% formic acid (pH<3) at room temperature. An aliquot was diluted tenfold and filtered through a polyvinylidene difluoride (PVDF) membrane with a 0.2-μm pore diameter. A 15-μl sample of the filtrate was injected into an high pressure liquid chromatography (HPLC) column. One bulb represented one replicate, and five replicates per treatment were performed.

Results and discussion

Effect of the initial cell density

The initial cell density significantly affected the SCV rate (v1) and the potential of the cells for producing embryos (v2, v3) (Fig. 1). Increasing the cell density from 1% to 8% decreased (v1). Consequently, a low cell density was the optimum condition for mass cell production. The initial cell density also affected the number of embryos per milliliter of SCV plated (v2). In contrast with (v1), (v2) and (v3) were highest when the cell density was 3–4% (Fig. 1). There may be several explanations for these effects of low initial cell densities of the sub-cultures. One is that the cultured cells altered the medium in a such a manner that allowed cell differentiation; thus, below the minimum density there were too few cells to affect this alteration (Ammirato 1983; Hari 1980) and the cells continued to propagate. (v1) was measured during the culture period at 14, 28, 42 and 56 days (data not shown). At initial cell densities of 1% and 2%, the optimal growth phase was reached within 42 days; at 3–5%, the optimal growth phase was reached within 28 days; at 8%, the optimal growth phase was reached within 14 days. Initial cell densities of 3–4%

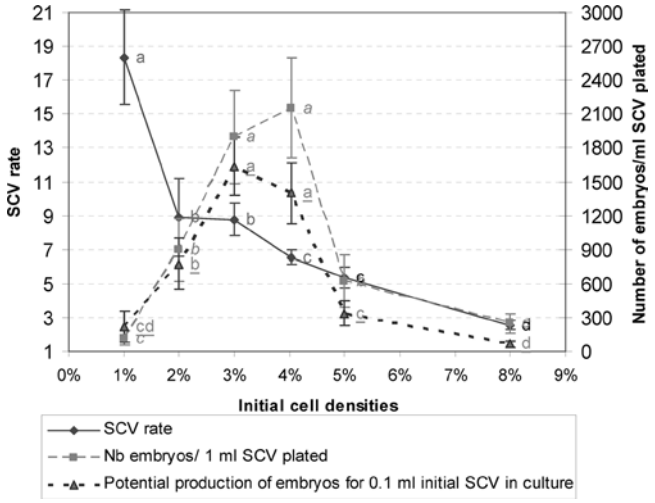

Fig. 1 Effect of initial cell densities 42 days post-subculture initiation of the cell suspension culture. *Letters* (*a*, *b*, *c*, *d*) on a same curve indicate homogeneous groups according to the *F*-test ($P<0.05$)

were particularly preferable both for long-term suspension culture before subculture (28 days) and for maintaining a better potential for later regeneration of embryos (v2, v3).

Effect of periodicity of medium renewal

The periodicity of medium renewal significantly affected the SCV rate (v1) and the production potential of embryos (v2, v3) (Fig. 2.). Thus, after 42 days of subculture, (v1) was higher at 14 days, (v2) was higher at 14 and 21 days and (v3) was higher at 14 days. Therefore, we determined that 14 days between media renewals was the best condition for both cell propagation and for the further regeneration of embryos (v2, v3).

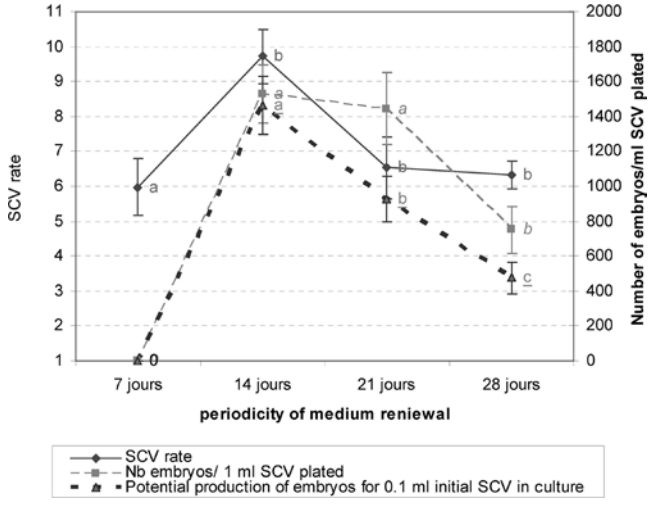

Fig. 2 Effect of periodicity of medium renewal, 42 days post-subculture initiation of the cell suspension culture. *Letters* (*a*, *b*, *c*) on a same curve indicate homogeneous groups according to the *F*-test ($P<0.05$)

Effect of periodicity of subculture

(v1) was measured after 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of subculture (data not shown) for the effect of different periodicities of medium renewal. At the best periodicity for medium renewal—14 days—we observed a reduction in the growth rate after 28 days of subculture despite renewal of the medium every 14 days. Therefore, we would recommend renewing the medium every 14 days subculturing the cell suspension every 28 days.

Effect of 2,4-D concentrations in the SM medium

The 2,4-D concentrations in the SM medium significantly affected the SCV rate (v1) and the potential for producing embryos (v2, v3) (Fig. 3.). Among the concentrations tested, 0.3 mg/l was effective in producing the greatest (v1), while the lowest response was obtained with 0.5 mg/l. With respect to (v2), 0.1 mg/l induced the highest potential, while 0.5 mg/l induced the lowest. Decreasing the 2,4-D concentration allowed the potential for further cell differentiation and regeneration capacity to be maintained. This result confirms the observations of Barandiaran et al. (1999), where a low concentration of 2,4-D increased the percentage of garlic explants showing cell multiplication through callus and regeneration. With respect to (v3), the lowest concentrations of 2,4-D (0.1 mg/l and 0.3 mg/l) gave results that were not significantly different, but still higher, than those obtained with 0.5 mg/l 2,4-D. We would hence recommend using the lowest concentration (0.1 mg/l) of 2,4-D to reduce the risks of somaclonal variation.

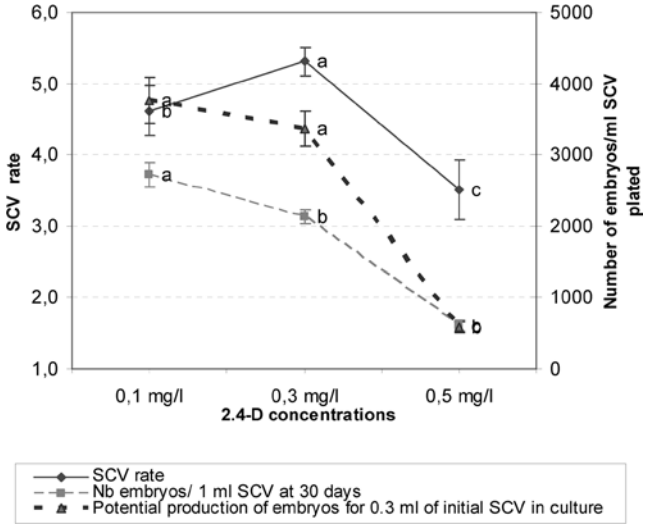

Fig. 3 Effect of 2,4-D concentration over three subsequent cycles of subculture after initiation, at a culture age of 28 days. *Letters* (*a*, *b*, *c*) on a same curve indicate homogeneous groups according to the *F*-test ($P<0.05$)

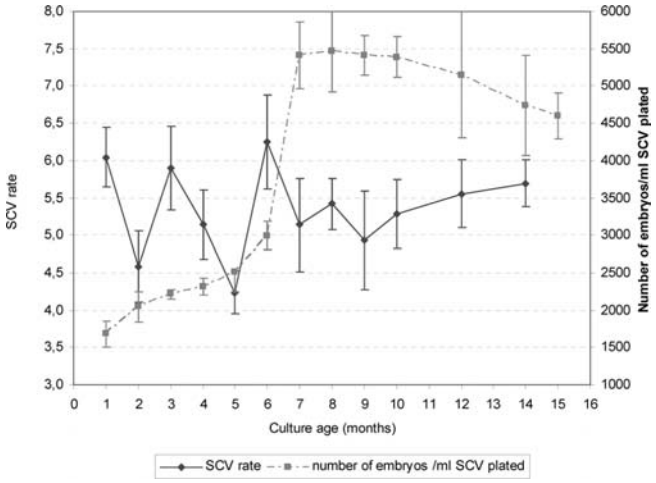

Fig. 4 Effect of the age of the cell suspension culture (from 1 to 15 months after initiation) on SCV rate and embryogenic competence

Effect of the age of the culture since initiation

The growth rate of the cell suspension culture (v1) was on average quasi similar with increasing culture age (up to 14 months), increasing about 4.5- to 6-fold within a 28-day period (Fig. 4). Nevertheless, for up to a 7-month period it fluctuated over a larger range (4.2–6.2), illustrating that the culture was very unstable. From 7 months onwards, the growth rate fluctuated over a narrow range, indicating a more stable condition.

Conversely, the number of embryos per milliliter SCV plated (v2) increased with the total age of the suspension culture until 7 months. It then remained stable from month 7 to month 12 and declined thereafter. For most species, the regeneration rate decreased as the age of the culture increased, which is the opposite situation to the present case (at least up to 7 months). Similar results have been obtained on garlic (Myers and Simon 1998) and onion (Phillips and Luteyn 1983) in the past. The specific culture conditions increased the number of shoots per explant with increasing callus age. Our results may be explained on the basis that our suspension culture conditions may be more effective than previous solid culture conditions at inducing regeneration and that these conditions continued to effect an improvement in the regeneration capacity within a 7-month period. This explanation may be based on the instability of the culture at the same period as reported above.

Regeneration of the cell suspension and the conversion of embryos into plantlets

Aliquots of cell suspension plated on semi-solid embryo production medium (EPM, Table 1) were able to produce numerous somatic embryos within 8 weeks. The first pro-embryos differentiated within 3 weeks after plating. They resembled large globular structures (1–2 mm) with a smooth surface due to the presence of an epidermis. The percentage of embryos converted into plantlets was 39.4%.

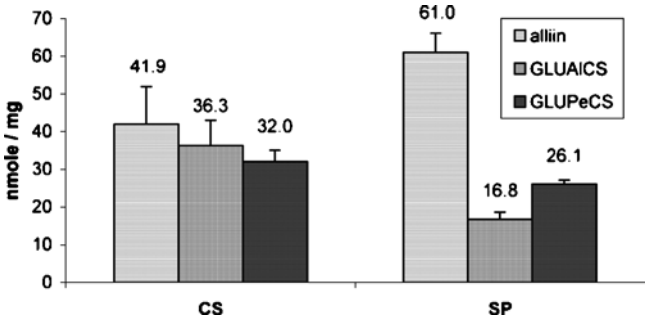

Fig. 5 Mean concentration (nmol/mg) of sulphur compounds in fresh CS and SP micro bulbs

The germination process occurred within 20 days. The germinated embryos were cultivated, and the plantlets were able to produce micro bulbs in vitro.

Comparison of the micro bulbs with respect to composition of the sulphur compounds

Differences were observed between (CS) and (SP) micro bulbs (Fig. 5). Alliin plus GLUAlCS represented 70% of the sulfur compounds for the two sources, but their distribution differed. In (CS) micro bulbs alliin decreased while GLUAlCS increased. In (SP) micro bulbs, alliin was more concentrated as opposed to the two dipeptides GLUAICS and GLUPeCS, which were less concentrated. The quantification of sulphur compounds underlined a difference in sulphur composition between the two types of garlic plant propagation. However, after two cycles of culture in the field, this difference was not maintained (data not shown).

Histological study

When the embryogenic calluses were cultivated in liquid medium, the friable embryogenic tissues (Fig. 6a and b) released embryogenic aggregates and single elongated or spherical cells. The cell aggregates multiplied and released new aggregates. After 28 days of culture in the liquid medium, a cell suspension culture was initiated (Fig. 6c). It consisted of a mixture of single cells up to aggregates of different sizes that were more than 1,200 μm (Fig. 6d). These were filtered through an 800-μm pore-sized sieve. The embryogenic cells were polyhedral and mostly isodiametric. The cytoplasm with small vacuoles was dense and rich in protein. Following plating on semi-solid EPM medium, these cells underwent a transverse division, resulting in two-, four- and multiple-cell structures that were determined to be pro-embryos. Cells of these pro embryos divided further and resulted in the formation of globular embryos. Following their transfer onto EDM medium, they later developed into complete and mature embryos (Fig. 6e and f) that showed a bipolar structure with shoot and root apices connected to an haustorium-like structure with vascular bundles, and entirely surrounded by an epidermis. These histological studies proved the embryogenic nature

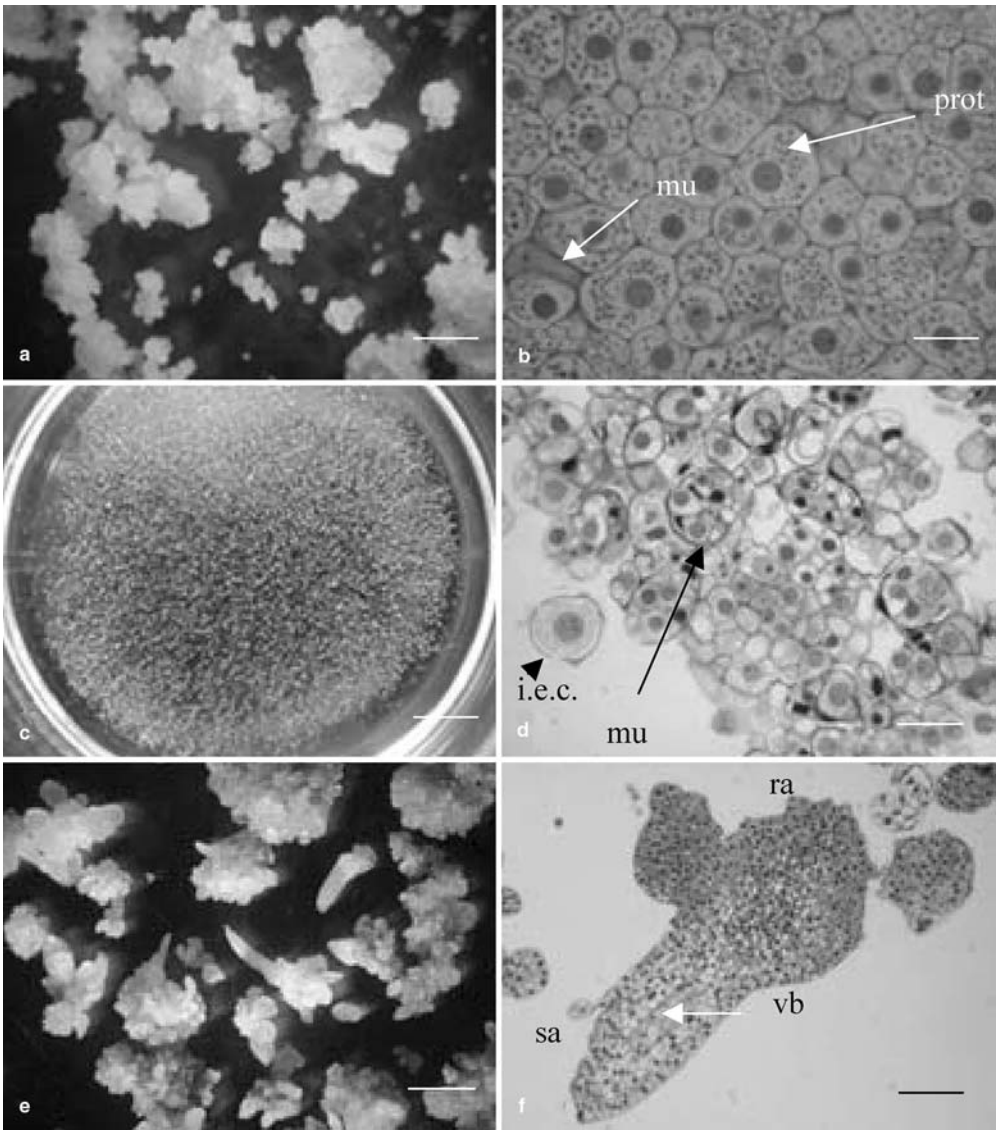

Fig. 6 Morphological (**a**, **c**, **e**) and histological (**b**, **d**, **f**) aspects of culture and regeneration of garlic embryogenic cell suspension. **a** Friable callus, **b** histological section of friable callus with cells embedded in mucilage (*mu*, *pink*) and showing storage protein grains (*prot*), **c** morphological aspect of a cell suspension culture of garlic, **d** histological section of the suspension culture showing isolated embryogenic cells (*i.e.c*) and small cell aggregates, **e** mature embryos on EDM medium 84 days after plating, **f** longitudinal section through a single embryo, showing the shoot (*sa*) and root (*ra*) apex, vascular bundles (*vb*). *Bar*: 0.025 mm (**b**), 0.5 mm (**c**), 0.05 mm (**d**), 1 mm (**a, e**), 5 mm (**f**)

of the process. A similar embryogenic processed occurs in other species, particularly in banana (Cote et al. 1996) or carrot (Yasuda et al. 2000).

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