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RESEARCH NOTE

Efficient plant regeneration from protoplasts of *Kalanchoe blossfeldiana* via organogenesis

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Abstract A simple and efficient protocol for plant regeneration from protoplasts of the potted plant *Kalanchoe blossfeldiana* Poelln. is reported. Mesophyll protoplasts were isolated from axenic leaves after a preculture. The enzymatic digestion of the tissue with a solution containing 0.4% Cellulase Onozuka R-10 and 0.2% Driselase yielded 6.0×10^5 protoplasts per gram fresh weight after density gradient purification. Protoplasts were cultured in the dark at an initial density of 1×10^5 protoplasts per milliliter in a liquid medium with 320 mM mannitol, 130 mM sucrose, 2.3 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 5.4 μM 1-naphthaleneacetic acid (NAA) and 2.2 μM 6-benzyladenine (BA). Cell wall regeneration was observed within 4 days of culture and cell division began after 5–7 days. When cultured in a liquid medium with 5.4 μM NAA and 8.9 μM BA, protoplast-derived colonies proliferated until small visible calli, and adventitious buds appeared after transfer to photoperiod conditions. Developed shoots were rooted on a solid medium supplemented with 0.6 μM indole-3-acetic acid (IAA) and successfully established under greenhouse conditions. The process required 4 months from isolation to rooted plants and the best conditions found gave a plant regeneration efficiency of 6.4 plants per 1×10^5 protoplasts. This is the first protocol reported for plant regeneration from protoplasts for a Crassulaceae family species.

Keywords *Kalanchoe blossfeldiana* · Plant regeneration · Protoplast culture

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Abbreviations

BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxy acetic acid
IAA	Indole-3-acetic acid
MES	2-(*N*-morpholino) ethane sulfonic acid
NAA	1-Naphthaleneacetic acid

Kalanchoe blossfeldiana, belonging to the Crassulaceae family and native to Madagascar, is a very popular pot plant thanks to its plentiful flowering and little need for care. *K. blossfeldiana* was introduced in Potsdam, Germany, in 1932 by Robert Blossfeld. Many mutants and hybrids were developed for florists use from the original *K. blossfeldiana* (Van Voorst and Arends 1982). Some of the earlier hybrids were not true from seed and had to be propagated by vegetative methods. Today almost all cultivars are propagated from terminal cuttings. Since 1988, *K. blossfeldiana* has been the most grown flowering pot plant in Europe with the total number of its annual production reaching approximately 65 million plants (Kalanchoe Growers Holland, http://www.kalanchoe.nl) and a annual cash value of 48.4 million euro in 2007 (Flower Council of Holland, http://www.flowercouncil.org). Considerable interest in the production of this ornamental plant leads to continuous development of cultivars with new traits so as to make the plant more attractive for the consumer and to reduce production costs (Christensen et al. 2008; Sanikhani et al. 2008).

In terms of ornamental plant breeding, tissue culture is a highly useful technique, as it makes possible to exploit genetic variation further away than classical breeding techniques do. While intraspecific variation represents the basis for somaclonal selection, extraspecific variation can be exploited through somatic hybridization, and both types

of variation can be profitable by means of genetic engineering (Deroles et al. 2002; Horn 2002). Somaclonal selection is a valuable breeding tool for ornamental plants. During indirect morphogenetic processes, genetic changes occur and the rate of these changes can be higher if the process is initiated from protoplasts. So it is expected to identify new mutants with desirable traits when an adequate number of protoplast-derived plants are evaluated. Any kind of change in plant architecture, leaf and flower morphology and size, etc. has potential value for the breeding of ornamental plants. *K. blossfeldiana* flowering constitutes one of the main traits for the success of this pot plant. Its wild counterparts (e.g. *K. beharensis*, *K. daigremontiana*, *K. laciniata*) stand out for its vegetative parts, especially for leaves with striking forms. Somatic hybridization between both kinds could generate a new variety combining beautiful leaves and flowers, which would have probably a high acceptance in the ornamental market. Plant genetic engineering in *K. blossfeldiana* can be performed through protoplast transformation and may contribute to the introduction of new traits, which could satisfy the consumer needs such as different growth habits, new flower patterns, colours and shapes and could manage as well important issues for producers of this ornamental crop such as a disease and pest resistance and abiotic stress tolerance.

There are some reports about tissue culture of *K. blossfeldiana* (Smith and Nightingale 1979; Schwaiger and Horn 1988; Sanikhani et al. 2006) and genetic transformation has been described (Aida and Shibata 1996, 1998; Christensen et al. 2008; Sanikhani et al. 2008). But regeneration from protoplasts has been reported neither for this species nor for a Crassulaceae species. Pierre and Queiroz (1986) reported the use of *K. blossfeldiana* protoplasts to study the Crassulacean Acid Metabolism (CAM) yet there was no protoplast culture reported. The objective of this research was to establish a plant regeneration system from protoplasts of *K. blossfeldiana*. A system of this kind launches the exploitation of several technologies with a practical approach, such as genetic manipulation, somatic hybridization and somaclonal variation, and provides unique systems for investigating most aspects of plant cell physiology and genetics (Davey et al. 2005).

Cultivar 'Tenorio' of *K. blossfeldiana* Poelln. was obtained from local nurseries (Viveros Vangarden, Valencia). Shoot segments with axillary buds were first washed thoroughly with water and then with 70% ethanol for 1 min. Explants were then surface-sterilised by immersion in a 2.5% sodium hypochlorite solution with 0.1% 7X-O-matic detergent (Flow Laboratories) for 20 min, rinsed three times with sterile distilled water and cultured on a solid basal medium a (BMa, Table 1) supplemented with 0.6 μM indole-3-acetic acid (IAA). Axenic plants obtained from shoots segments were propagated every 2 months to the same medium, maintained as in vitro stocks plants and used as a source of axenic leaf explants. All cultures were incubated at 25°C under a 16 h fluorescent light of 60 μmol m^{-2} s^{-1} intensity (GroLux, Sylvania). Mesophyll protoplasts (pps) were isolated from leaves of 2 month-old axenic plants. Leaf explants were excised from axenic plants and cultured for 2 days in a solid

Table 1 Culture procedures used to regenerate plants from protoplasts of *K. blossfeldiana*

Culture steps	Basal medium	Other components	Duration
Axenic plant culture	Solid BMa	0.6 μM IAA	2 months
Leaf explants preculture	Solid BMa	5.4 μM NAA + 8.9 μM BA	2 days
Protoplast isolation	Liquid WM	0.4% cellulase + 0.2% driselase	4 h
Protoplast purification	Liquid FM		
Culture of protoplasts	Liquid BMb	2.3 μM 2,4-D + 5.4 μM NAA + 2.2 μM BA	30 days
Culture of protoplast-derived colonies	Liquid BMb	5.4 μM NAA + 8.9 μM BA	15 days
Culture of small calli	Liquid BMc	5.4 μM NAA + 8.9 μM BA	15 days
Culture of green calli	Solid BMc	5.4 μM NAA + 8.9 μM BA	15 days
Culture of calli with buds	Solid BMa	5.4 μM NAA + 8.9 μM BA	15 days
Culture of shoots	Solid BMa	0.6 μM IAA	30 days
Plants			30 days

WM (washing medium): MS macroelements, 400 mM mannitol, 100 mM glycine, 14 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM MES. FM (flotation medium): MS macroelements, 650 mM sucrose, 50 mM glycine, 14 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM MES. BMa (basal medium a): MS mineral solution, 90 mM sucrose, 0.6 mM myo-inositol, 3 μM thiamine-HCl, ST vitamins. BMb (basal medium b): macronutrients (5 mM NH4NO3, 15 mM KNO3, 3 mM CaCl2·2H2O, 1.5 mM MgSO4·7H2O, 0.5 mM KH2PO4), MS micronutrients, 320 mM mannitol, 130 mM sucrose, 28 mM myo-inositol, 25 mM xylitol, 0.3 mM ascorbic acid, 0.05 mM adenine hemisulfate, 0.5 mM MES, SH vitamins. BMc (basal medium c): MS mineral solution, 210 mM mannitol, 90 mM sucrose, 0.6 mM myo-inositol, SH vitamins. All solutions were adjusted to pH 5.7. Media were autoclaved, except for FM and enzymatic solution that were filter sterilised. Cultures were supplemented with 200 mg/l filter sterilised ticarcillin/clavulanate mixture (Duchefa Biochemie BV). Solid media contained 8 g l^{-1} agar (Agar Bacteriologico Europeo, Pronadisa). Fresh medium was added to liquid cultures every week. MS mineral solution (Murashige and Skoog 1962); SH vitamins (Shahin 1985); ST vitamins (Staba 1969)

BMa medium supplemented with 5.4 μM 1-naphthaleneacetic acid (NAA) and 8.9 μM 6-benzyladenine (BA) under a 16 h photoperiod. One gram of fresh weight (gfw) of precultured leaf explants was chopped into small pieces and immersed in 10 ml of an enzymatic solution containing 0.4, 0.8 or 1.2% (w/v) Cellulase Onozuka R-10 (Duchefa Biochemie BV) and 0.2% (w/v) Driselase (Sigma–Aldrich) dissolved in the washing medium (WM, Table 1) and digestion was carried out at 25°C at 40 rpm for 4 h in the dark. The resulting mixture was passed through a 63 μm nylon sieve and the filtrate was centrifuged (100 g, 10 min). The pellet was carefully resuspended in 6 ml flotation medium (FM, Table 1). Protoplasts were purified by density gradient centrifugation (50 g, 10 min) after layering 1 ml WM upon the flotation medium. Floated protoplasts were transferred into a new tube and washed with 8 ml WM. Four cell counts for each experiment were carried out in a microscope counting chamber to estimate the yield (expressed as number of pps per gfw). After yield estimation, protoplasts were centrifuged (100 g, 10 min), the pellet was resuspended in CM1 medium, protoplasts were diluted up to 1×10^5 pps ml^{-1} and cultured in 50 mm diameter Petri dishes containing 2.5 ml medium each. The CM1 medium for protoplast culture consisted of the basal medium b (BMb, Table 1) supplemented with 2.3 μM 2,4-dichlorophenoxy acetic acid (2,4-D), 5.4 μM NAA and 2.2 μM BA. Protoplast culture was carried in liquid medium in the dark adding every 7 days fresh medium. The presence or absence of cell wall regeneration was examined under a microscope after isolation and during culture. After 30 days, protoplast-derived colonies were cultured for further proliferation using two techniques: liquid culture (2.5 ml protoplast-derived colonies suspension per Petri dish) and solid culture (2.5 ml of 0.7% agarose embedded protoplast-derived colonies per Petri dish). In both techniques, cultures were kept in the dark and three different regulator combinations were tested: CM1 medium (as described above), CM2 medium (BMb with 5.4 μM NAA and 8.9 μM BA) and CM3 medium (BMb with 2.2 μM BA). After 15 days, liquid CM4 medium was added to the proliferated calli and cultures were transferred to a 16 h photoperiod. CM4 medium consisted of basal medium c (BMc, Table 1) supplemented with 5.4 μM NAA and 8.9 μM BA. Green calli appeared and they were cultured first in a solid CM4 medium and later on a solid CM5 medium (BMa with 5.4 μM NAA and 8.9 μM BA). Shoots developed were separated from organogenic calli and cultured in glass jars with 40 ml solid BMa supplemented with 0.6 μM IAA. After 1 month rooted plantlets were carefully removed from the jars, roots were washed thoroughly with water to remove traces of agar and then transferred to plastic pots or plug trays containing coco peat or peat moss as substrate. The plantlets were cultured in growth chambers at 25°C with 16 h photoperiod and initially covered with a transparent plastic to maintain high humidity. After 1 week plastic was taken away and 1 month later plants were transferred to a greenhouse with the same temperature and photoperiod. All experiments were repeated three times. Data variability was expressed as the mean ± standard error (SE). Means differing significantly were compared using Duncan's multiple range test at a 5% probability level.

In the first experiment, protoplasts were isolated from leaves of axenic plants (Fig. 1a) without preculture. Different concentrations of cellulase (0.4, 0.8 and 1.2%) and the presence of driselase (0.2%) were tested for protoplast isolation. Enzymatic digestion (Fig. 1c, d) was performed for 4 h and protoplasts were purified by density gradient centrifugation (Fig. 1e), using as flotation medium FM 0.60 medium, with 550 mM sucrose and 50 mM glycine, and yield was estimated. The results indicated that maximum protoplast yield (2.0×10^5 pps gfw^{-1}) was obtained using the enzyme solution containing 0.4% cellulase and 0.2% driselase (Table 2). Enzymatic solutions without driselase left plant tissue intact even if the duration of the digestion was expanded until 12 h. When enzymatic solutions with driselase were left more than 6 h tissue and protoplasts were severely damaged. Other experiments (data not shown) revealed that the addition of other enzymes, such as pectinase, did not improve the best yield obtained. The protoplasts obtained were round-shaped with a diameter between 30 and 50 μm. Some protoplasts had chloroplasts distributed in a homogenous way through the cytoplasm and some others seemed vacuolated, with chloroplasts concentrated in one zone of cytoplasm.

In the second experiment, prior to enzymatic digestion, explants from axenic leaves were precultured for 2 days in a solid BMa supplemented with 5.4 μM NAA and 8.9 μM BA under a 16 h photoperiod (Fig. 1b), and yield obtained was compared with the one from axenic leaves without preculture. The enzymatic solution used was cellulase 0.4% and driselase 0.2% and digestion was performed for 4 h. There was no difference in protoplasts yield (1.8×10^5 pps gfw^{-1} without preculture and 2.0×10^5 pps gfw^{-1} with preculture), but there was difference in the type of protoplasts obtained: without preculture homogenous and vacuolated protoplasts were obtained as described before and with preculture a third kind of protoplasts appeared, with less chloroplasts and a dense cytoplasm. Next experiments were conducted with precultured axenic leaves.

When protoplast isolation from *K. blossfeldiana* was compared with isolation conducted in the same conditions for other species, it was observed that the amount of protoplasts after enzymatic digestion was higher in *K. blossfeldiana* but was much lower after the protoplast purification process. Some experiments were carried to culture protoplasts without purification, but that was not possible and all cells died. Finally, it was found that if purification was performed with a flotation medium with a

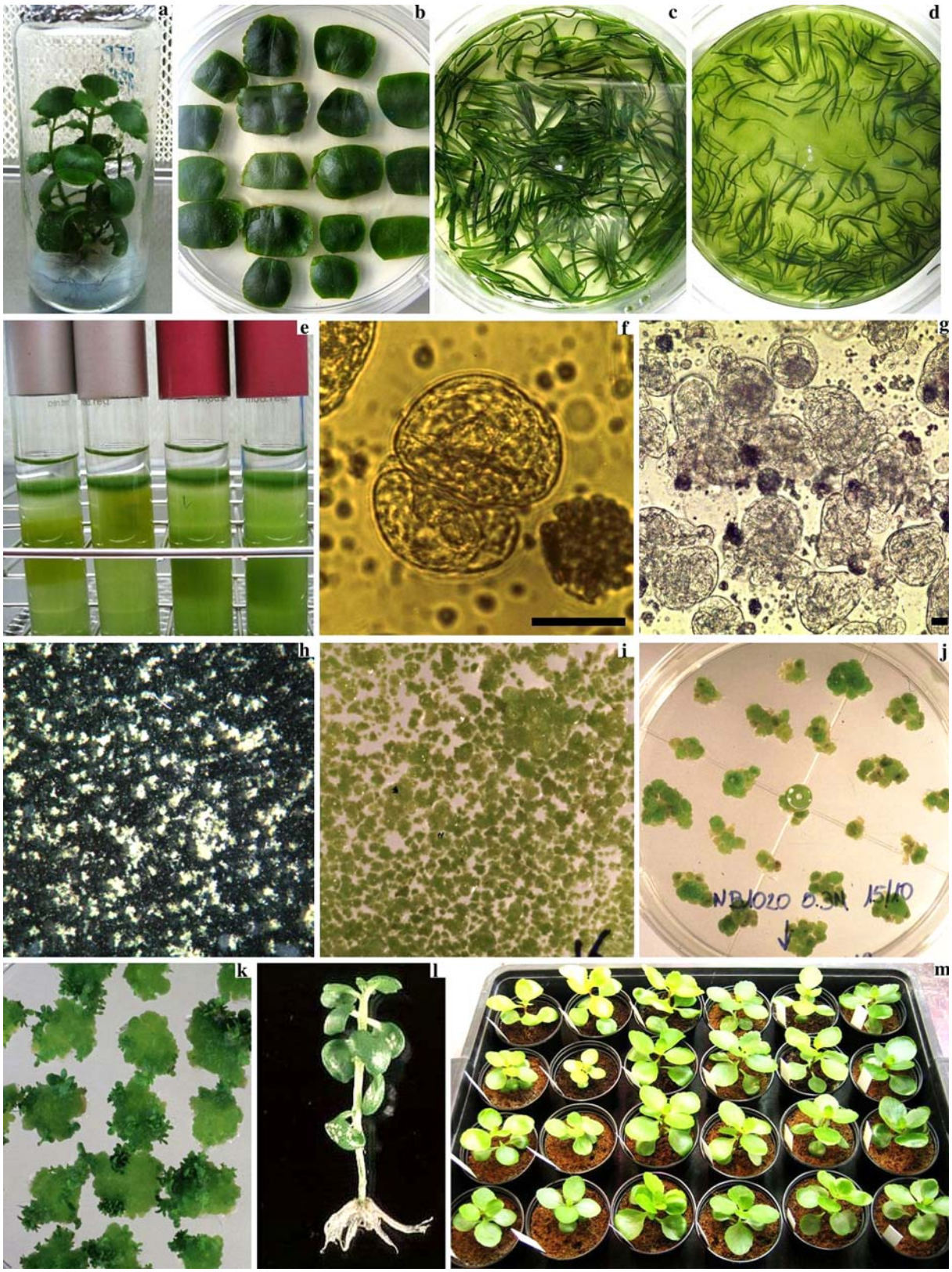

Fig. 1 Plant regeneration from protoplasts of *K. blossfeldiana*. **a** Axenic plant used as source of leaf explants; **b** leaf explants preculture; **c** leaf tissue before and **d** after enzymatic digestion; **e** protoplast purification; **f** first cell divisions; **g** protoplast-derived colonies; **h** visible small calli; **i** green calli; **j** organogenic green calli; **k** calli with shoots; **l** rooted shoot; **m** plant acclimatization. *Bar* = 25 μm

Table 2 Effect of enzyme solution composition on protoplast yield

Cellulase conc. (% w/v)	Driselase conc. (% w/v)	Yield ($\times 10^5$ pps gfw^{-1})
0.4	0	0[a]
0.8	0	0[a]
1.2	0	0[a]
0	0.2	1.1 ± 0.3[b]
0.4	0.2	2.0 ± 0.2[c]
0.8	0.2	0.1 ± 0.0[a]
1.2	0.2	0.1 ± 0.0[a]

Different letters indicate significant differences ($P = 0.05$) by Duncan's multiple range test

Table 3 Effect of medium on the culture of protoplast-derived colonies

Culture system	Medium culture	PRE[1]
Solid	CM1	1.0 ± 0.6[ab]
	CM2	4.6 ± 0.2[c]
	CM3	0.0 ± 0.0[a]
Liquid	CM1	4.8 ± 0.0[c]
	CM2	6.4 ± 0.4[d]
	CM3	1.9 ± 0.1[b]

[1] PRE (plant regeneration efficiency): number of plants regenerated per 1×10^5 initial protoplasts

Different letters indicate significant differences ($P = 0.05$) by Duncan's multiple range test

higher sucrose concentration (FM 0.70 medium, with 650 mM sucrose and 50 mM glycine) protoplast yield, 6.0 ± 0.5 10^5 pps gfw^{-1}, was triplicated with respect to the flotation medium usually employed (FM 0.60 medium, with 550 mM sucrose and 50 mM glycine), which gave 1.8 ± 0.2 $\times$ 10^5 pps gfw^{-1} under the same conditions.

Purified protoplasts were cultured at an initial density of 1×10^5 pps ml^{-1}. Cell wall regeneration was observed within 4 days of culture and cell division began after 5–7 days (Fig. 1f). Liquid CM1 medium was added to cultures every week and protoplast-derived colonies began to grow at 15 days after isolation. The osmotic potential achieved using 320 mM mannitol and 130 mM sucrose (CM1 medium) was optimal for *K. blossfeldiana* protoplast culture. Some variations introduced in media for a better osmotic adjustment did not improve protoplast culture (data not shown). Macronutrient solution used in media for *K. blossfeldiana* protoplast culture (BMb basal medium, Table 1) was developed for other species in which MS or B5 (Gamborg et al. 1968) macronutrients did not allowed protoplast culture. After 30 days, protoplast-derived colonies were in extensive proliferation (Fig. 1g) and then two culture systems (liquid and solid culture) and three regulator combinations (CM1, CM2 and CM3 media) were tested. Colony growth was higher in liquid media than in solid system (Table 3). With respect to the kind of regulator combinations, all media tested allowed proliferation and small calli (1–3 mm diameter) were obtained, but at different rates, with CM2 medium reaching the highest rate and CM3 the lowest (Table 3). In the case of solid CM3 culture, growth was so slow that colonies were unable to continue and cultures finally died. Liquid CM2 media was the best culture as it yielded 6.4 plants per 1×10^5 initial protoplasts.

The visible calli originated had a cream colour (Fig. 1h), liquid CM4 medium was then added and cultures were transfer to 16 h photoperiod. In 15 days calli proliferated and turned green (Fig. 1i). These ones were subcultured to a solid CM4 medium and adventitious buds emerged (Fig. 1j). A subsequent culture on CM5 medium (BMa with 5.4 μM NAA and 8.9 μM BA) allowed the development of adventitious buds into shoots (Fig. 1k). In some cases, manipulation of small calli with forceps or scalpel tip generated some browning in calli surface after 1–2 days, but this fact did not interfere neither with the growth nor with the organogenic ability of calli. Shoots developed long enough (more than 1 cm) were excised from organogenic calli and cultured in BMa supplemented with 0.6 μM IAA. After 1 month all plant had developed roots (Fig. 1l) and they were acclimatized first in a growth chamber (Fig. 1m) with high humidity and later on a greenhouse, where they flowered normally.

In the present work, *K. blossfeldiana* plants were regenerated from mesophyll protoplasts isolated from axenic plants. The procedure from protoplast isolation to plant regeneration is outlined in Table 1 and Fig. 1. The best protoplast yield obtained was 6.0×10^5 pps gfw^{-1} and the best plant regeneration efficiency was 6.4 plants per 1×10^5 pps. Although protoplast yield was not as high as in other species (Nassour and Dorion 2002), globally the process arose very efficient as it produced 38.4 plants gfw^{-1}. Glycine was added to the washing and flotation media used in the present protocol. Glycine is one of the well-known osmoprotectants, which stabilizes membranes during osmotic stress, and more stable protoplasts after isolation have been reported when glycine was used (Orczyk and Malepszy 1985). In our laboratory we have confirmed this effect in protoplasts from other species and we use it routinely in protoplasts handlings. Leaf preculture before protoplast isolation permitted obtaining a third type of protoplast in addition to the two types found when isolation was performed with leaves without preculture. It was not known if that kind was more able to divide. This kind of protoplast was thought to be derived from the cells starting to dedifferentiate in the preculture. There are reported differences in the type of protoplast observed depending on the source material. In rice, protoplasts isolated from cell

cultures were densely cytoplasmic immediately after isolation (Tang et al. 2001). In mesophyll protoplasts of rapeseed, sub-populations were characterised as vacuolated (containing few or no chloroplasts and a large vacuole) or chloroplastic (containing chloroplasts and no large vacuole) (Millam et al. 1991). As far as we know, this is the first protocol reported for plant regeneration from protoplasts for a Crassulaceae family species. The immediate application of this protocol is a program of somaclonal selection for *K. blossfeldiana*, as high number of plants can be regenerated. However, somatic hybridization and genetic engineering (nuclear or chloroplastic) can be addressed with the basis of this protocol.

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