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Thidiazuron-induced plant regeneration from protoplasts of *Vicia faba* cv. Mythos

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Summary. Protoplasts of 10 cultivars of *V. faba* were isolated from etiolated shoot-tips and tested for their regeneration capacity. After purification, protoplasts were embedded in sodium alginate and cultivated in the medium of Kao and Michayluk (1975) containing 0.5 $mg \cdot l^{-1}$ of each 2,4-dichlorophenoxyacetic acid, naphthylacetic acid and 6-benzylaminopurine. Depending on cultivar, division frequencies of up to 40% were obtained. Six weeks after embedding, protoplast-derived calluses were transferred to Gelrite-solidified media with different combinations of growth regulators. A two step protocol (auxin high/low) was tested for its ability to induce somatic embryogenesis. The formation of globular structures was observed, but no embryo formation could be achieved. In contrast, cultivation of protocalluses on medium supplemented with thidiazuron resulted in shoot development in cultivar Mythos. To generate mature plants, the shoots were grafted onto young seedlings. In order to optimize the *in vitro*-conditions, different concentrations of thidiazuron alone or in combination with naphthylacetic acid were tested, showing that an increase of thidiazuron and the addition of naphthylacetic acid positively affects both the viability of protocalluses and the regeneration frequency.

Abbreviations: 2,4-D=2,4-dichlorophenoxyacetic acid; BA=5-benzylaminopurine; GA_3=gibberellic acid; IBA=indole-3-butyric acid; Kin=kinetin; KM=Kao and Michayluk; MS=Murashige and Skoog; NAA=naphthylacetic acid; TDZ=thidiazuron; Zea=zeatin;

Introduction

Today protoplast technology is well established in many plant species and routinely used for cell manipulation such as somatic hybridisation and direct gene transfer (Puite 1992). However, in grain legumes the regeneration of plants from single cells still remains difficult. Up to now 13 reports have been published describing the successful recovery of plants from protoplasts: in *Vigna aconitifolia* (Shekawat and Galston 1983; Köhler et al. 1987), *Psophocarpus tetragonolobus* (Wilson et al. 1985), *Pisum sativum* (Puonti-Kaerlas and Eriksson 1988; Lehminger-Mertens and Jacobsen 1989; Böhmer and Jacobsen 1994), *Glycine canescens/clandestina* (Newell and Luu 1985; Hammatt et al. 1987; Myers et al. 1989), *G. argyrea* (Hammatt et al. 1989), and *G. max* (Wei and Xu 1988; Dhir et al. 1991a and 1992a).

Relative to the great economical importance of grain legumes this appears to be a small number, especially when one compares it to the numerous reports describing plant regeneration from multicellular explants (see Parrott et al. 1992). A "follow up" of the published data, i.e. the exploitation of the presented protocols for protoplast regeneration in transformation experiments or in somatic hybridisation has occurred only occasionally (Köhler et al. 1987; Hammatt et al. 1992; the reports of Dhir et al. describing the transformation of soybean in 1991(b) and 1992(b) were retracted by Widholm (1993a, b)). This indicates that even when the appropriate *in vitro*-conditions for plant recovery from protoplasts have been found, regeneration of grain legumes generally remains problematic and is often difficult to reproduce.

In *Vicia faba* as well as in all other *Vicia* species which have so far been tested in tissue culture only callus was obtained from protoplasts (Kao and Michayluk 1975; Binding and Nehls 1978; Donn 1978; Röper 1980). In the present study we followed two different approaches to initiate plant regeneration: (i) Induction of somatic embryogenesis by exposing protoplast-derived calluses to media containing "strong" auxins (picloram, dicamba, 2,4-D) followed by a decrease in auxin strength; (ii) induction of direct shoot regeneration by exposing protocalluses to cytokinins and thidiazuron, a phenyl-urea herbicide exhibiting a strong cytokinin-like activity (Mok et al. 1982).

In our report, we describe the development of a culture system which allows the reproducible regeneration of fertile plants from protoplasts in *V. faba* cv. Mythos. The key step in the procedure is the cultivation of protoplast-derived calluses on thidiazuron-containing media.

Material and methods

Plant material. Seeds of 10 cultivars of *V. faba* (see Table 2) were surface sterilized for 1 min in 70% (v/v) ethanol, 10 min in a 5% (v/v) sodiumhypochlorite solution and then rinsed 4 times with sterile tap-water. After soaking for 6 - 8 hours, the seeds were transferred into 250 ml jars (3-4 seeds each) containing ½ concentrated MS-basal medium (Murashige and

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Skoog 1962) solidified with 0.8 % agar (Merck) and kept for germination in the dark at 24°C. Shoot-tips from the etiolated seedlings (hook plus approx. 2 cm of the epicotyl, see Fig. 1a) served as the source for protoplasts. After cutting the primary shoot, secondary shoots which arise from the cotyledonary node during the following weeks were also used for protoplast isolation without a decrease in protoplast yield.

Protoplast isolation. Shoot tips were cut in 0.5-1 mm segments and incubated in an enzyme solution (1.5-2 g tissue per 50 ml) containing 5% cellulase TC (Serva), 1% pectinase (Serva), 1% macerozyme R10 (Yakult), 1% hemicellulase (Sigma), 8 mM $CaCl_2$ and mannitol to bring the osmolality to 500 mOsm·kg^{-1} (pH 5.5). Digestion was performed in 250 ml flasks for 14 h in the dark at 25°C under continuous rotation (4 rpm) in a Cell Production Roller Apparatus (Bellco/USA). After incubation the protoplast solution was filtered through two layers of steel sieves (125 and 63 µm mesh size) followed by centrifugation for 8 min at 150 x *g*. Subsequently, the protoplasts were washed twice in 0.2 M $CaCl_2$ (resulting in precipitation of cell debris from burst protoplasts) and were filtered through a 60 µm nylon-filter, giving a pure solution of viable protoplasts. After centrifugation at 150 x *g* for 8 min, protoplasts were resuspended in 0.48 M mannitol (500 mOsm·kg^{-1}). The purified protoplasts were kept in mannitol for 12 h at 4°C in the dark.

Protoplast culture. Different conditions were tested for the initial culture of isolated protoplasts: (i) culture in liquid medium (aliquots of 6 ml of protoplast suspension in 60 x 15 mm plastic Petri-dishes), (ii) embedding in 0.8% (w/v) Seaplaque-agarose (using the protocol of Shillito et al. 1983) and (iii) embedding in sodium alginate (procedure see below). Protoplast density was adjusted to 2.5x10^5·ml^{-1}. KM-medium (Kao and Michayluk 1975) containing 0.5 mg·l^{-1} of each BA, NAA and 2,4-D (KM7-medium) was generally used as culture medium. During the first ten days protoplasts were cultured in KM7 adjusted to 500 mOsm·kg^{-1} with mannitol. The osmolality was subsequently reduced by diluting the original medium with osmoticum-free KM7 in a ratio of 1:1 at day 10, 20, 30 and 40. Protoplasts were cultured at 25°C in the dark.

For embedding in sodium alginate, equal volumes of the protoplast suspension and a 0.4 M mannitol solution containing 2.0, 2.6, or 3.0% sodium alginate (to give final concentrations of 1.0, 1.3 and 1.5% respectively, see Table 1) were mixed and dispersed on agar-plates (0.5 ml aliquots) containing 0.4 M mannitol and 0.02 M $CaCl_2$ for polymerisation. The alginate disks were subsequently transferred into 60 x 15 mm plastic Petri-dishes containing 4 ml KM7-medium. The influence of different alginate types (A2033+A2158 of Sigma/Aldrich and E401 of Roth, 76185 Karlsruhe, Germany) was tested in preliminary experiments (see Table 1). Due to its superior properties, in all further experiments Roth-alginate E401 in a final concentration of 1.0% was used for protoplast embedding.

Cell division frequency was calculated 15 days after protoplast isolation and is defined as the percentage of cells that have divided at least once. Plating efficiency was calculated after 35 days and is defined as the number of developing protocalluses per number of protoplasts plated.

Regeneration. Since protoplasts of *V. faba* failed to divide in liquid medium as well as after embedding in seaplaque-agarose, only protocalluses derived from alginate-embedded protoplasts could be subjected to regeneration studies. Protocalluses were released from alginate 6-8 weeks after protoplast isolation by incubation for 30 min in a 20 mM sodium-citrate solution for depolymerisation of the alginate matrix. After centrifugation (100 x g, 3 min) calluses were washed twice in distilled water and transferred to MS-medium (solidified with 0.25% Gelrite/Roth, pH adjusted to 5.7 prior to autoclaving) containing different combinations of growth regulators:

For the induction of somatic embryogenesis (according to the protocol of Pickardt et al. 1989) protoplast-derived calluses were transferred to MS-medium (3% sucrose) supplemented with either 0.1/0.5/1/2/4/10/20/40/60/80 mg·l^{-1} picloram, or dicamba or 2,4-D (high-auxin stage). Cultures were kept in the dark at 25° C. After four weeks calluses were transferred to MS-medium (1.5% sucrose) containing 1 mg·l^{-1} NAA (low-auxin stage) and incubated at 25°C under a 16 h photoperiod (79 µmol·m^{-2}·s^{-1}). Calluses were subcultured every four weeks on this medium. In addition, part of the calluses were further subcultured on the above mentioned high-auxin media for a minimum of five months, to compare high/low-auxin vs. high-auxin permanantly. On each of the above media 100 (high/low-auxin) and 50 protocalluses (high-auxin alone) were tested.

For shoot regeneration protocalluses were transferred to MS-medium (3% sucrose) supplemented with 50 mg/l casein hydrolysate and different combinations and concentrations of growth regulators (see Table 3 and Fig. 2). Calluses were incubated at 25°C, a 16 h photoperiod and a light intensity of 79 µmol·m^{-2}·s^{-1} and were subcultured every four weeks. In order to test the influence of TDZ and NAA (Fig. 2) on survival rates and regeneration frequencies 100-150 calluses and 40-60 calluses, respectively, were plated on each medium.

Mature plants were generated by grafting shoots with a length of 0.5 - 2 cm onto 5-6 days old etiolated seedlings. Grafting was done as follows: The epicotyl of the seedling was cut off 1.5 - 2 cm above the cotyledonary node. The remaining stem was then cut longitudinally approximately 1 cm deep. The stem of an appropriate shoot was cut in the shape of a wedge and inserted into the shoot between the split epicotyl of the seedling. Finally, all secondary shoots which arose from the cotyledonary node of the seedling during the following days were removed. After leaf development had been established, the grafted plants were transferred to soil and placed in the greenhouse.

Results

Isolation of protoplasts

One of the main problems in protoplast isolation and cultivation of *V. faba* is the lethal browning of cells caused by polyphenolic oxidation (a phenomenon generally observed in tissue culture of *V. faba*). Protoplasts could occasionally be isolated from green leaves of *V. faba* cvs. Herz Freya and Mythos, however, in most experiments cells died after browning. In contrast, shoot tips from etiolated seedlings (Fig. 1a) proved to be a suitable source for protoplast isolation. Protoplast yields from shoot tips digested for 14-16 hours in a high concentrated enzyme solution ranged from 1.3 to 4.7x10^6·g^{-1} fresh weight depending on cultivar/line (Table 2). A shorter period of digestion decreased the yield of protoplasts, whereas extended periods of digestion above 16 hours decreased the number of viable cells, probably due to the accumulation of toxic phenolic compounds.

After separation of protoplasts from undigested tissue by filtering through 125 and 63 µm steel sieves a further purification could be achieved by a treatment with 0.2 mM $CaCl_2$. Cell debris from burst protoplasts formed precipitates under these conditions (Fig. 1b), probably due to the action of divalent calcium ions. These precipitates could be removed by repeated filtration which resulted in a pure protoplast solution (Fig. 1c).

Table 1. Influence of protoplast culture method on division frequency (after 15 days) and plating efficiency (after 35 days) of *V. faba* cv. Mythos

Protoplast culture		Division frequency (%)*	Plating efficiency (%)*
Liquid medium		0	0
Embedding in agarose		0	0
Embedding in alginate:			
Sigma A2033	1.5%	4.24±0.92	0.01±0
Sigma A2158	1.5%	6.31±1.11	0.04±0.01
Roth E401	1.5%	10.20±1.64	0.19±0.04
	1.3%	35.29±4.89	0.36±0.06
	1.0%	39.86±4.09	0.56±0.09

*mean value and standard deviation (σ_{n-1}) of three experiments

Table 2. Division frequency (%) and plating efficiency (%) in different cultivars of *V. faba* after 15 and 35 days respectively (plating density 2.5×10^5·ml^{-1})

Cultivar	Origin	Protoplast yield per g f. w.*	Division frequency (%)**	Plating efficiency (%)**
Mythos	NPZ***/Germany	4.7×10^6	39.9±4.09	0.56±0.09
Carola	NPZ/Germany	1.9×10^6	7.8±5.3	0.02±0.02
Caspar	Cebeco/Netherlands	2.2×10^6	17.1±5.3	0.08±0.03
Herz Freya	Josef Herz/Germany	3.6×10^6	36.3±14.0	0.33±0.24
Kristall	Lochow Petkus/Germany	3.7×10^6	13.6±12.6	0.10±0.10
Piccolo	NPZ/Germany	3.3×10^6	28.1±2.2	0.24±0.04
Topas	NPZ/Germany	1.9×10^6	4.9±1.6	0.01±0.00
Troy	NPZ/Germany	1.7×10^6	4.4±0.7	0.03±0.02
Dreifach-Weiße	Sperling/Germany	2.4×10^6	15.0±0.6	0.24±0.01
Albatross	NPZ/Germany	2.3×10^6	13.1±3.7	0.15±0.05

*fresh weight; ** mean value and standard deviation (σ_{n-1}) of a minimum of three experiments; *** Norddeutsche Pflanzenzucht

Protoplast culture

Cultivation in liquid media or agarose-embedding resulted in an intensive browning of protoplasts. Cell death rapidly ensued and was probably caused by phenolic oxidation. Cell divisions could not be observed under either of these conditions (Table 1). In contrast, in protoplast cultures embedded in alginate, the first divisions occurred after 6-7 days. By day 15 almost 40% of cells had divided (Fig. 1d) and after five weeks a plating efficiency of 0.56% was achieved when protoplasts were embedded in alginate type E401 of Roth in a final concentration of 1% (Table 1). Under these conditions a value of 0.56% represents the maximal number of colonies (approx. 600) which can develop on an alginate disk of an area of 5-6 cm^2, giving a lawn of colonies as is shown in Fig. 1e. Attempts to obtain a higher plating efficiency by decreasing the number of embedded protoplasts failed, since an initial cell density of 2.5×10^5 protoplasts·ml^{-1} was necessary for initiation of cell division (data not shown).

Higher concentrations of alginate (1.3 and 1.5%) or the use of alginate type A2033 and A2158 of Sigma resulted in lower division frequencies and plating efficiencies. Therefore, in all further experiments Roth-alginate type E401 in a concentration of 1% was used.

Six to eight weeks after protoplast isolation protocalluses were released from alginate by incubation with sodium-citrate for depolymerisation of the alginate matrix and transferred to solid medium (Fig. 1f).

In order to test the genotypic influence on the regeneration capacity, protoplasts from ten different cultivars of *V. faba* were isolated and cultured according to the protocol which has been established for cultivar Mythos. Dependent on cultivar, cell division frequencies between 4.4 and 36.3% and plating efficiencies ranging from 0.01 up to 0.37% were achieved (Table 2).

Plant regeneration

A prerequisite for testing a high number of different media and culture conditions are sufficiently healthy and well growing cultures. However, throughout our studies we were facing the problem of lethal browning of tissue during the subculture of calluses. In many experiments the number of calluses tested declined continuously, caused by phenolic oxidation during cultivation. The extent of lethal browning, however, was genotype-dependent. Since minor losses were observed in cv. Mythos, regeneration experiments were mainly untertaken with this cultivar. Therefore, in the following observations and results we always are referring to cv. Mythos.

All attempts to induce somatic embryogenesis were unsuccessful. 150 calluses were tested on each of the 30 different media, with or without a subsequent phase with lowered auxin strength. Generally, a soft, friable and yellow coloured callus developed on auxin media. The level of phenolisation was influenced by the auxin concentration: cultivation above 5 mg·l^{-1} 2,4-D or dicamba and above 20 mg·l^{-1} picloram led to cell death. Globular structures (Fig. 1g) occasionally arised from callus during the low-auxin stage, when callus was induced on MS-medium supplemented with 1-4 mg·l^{-1} 2,4-D or picloram, but no further development could be observed.

For the induction of shoot morphogenesis, 48 combinations/concentrations of different growth regulators were tested (see Table 3). In these experiments we found an overall survival rate of approx. 20% in cv. Mythos after ten months of culture. The viability of calluses varied greatly between individual experiments, thus comparisons of different culture conditions concerning the survival rate were only possible when protocalluses from the same protoplast isolation experiment were tested (see below, Fig. 2 a+b).

Shoot morphogenesis was initiated only on TDZ-containing medium under the given conditions. The time until the start of shoot development varied between 4 and more than 10 months in individual calluses. Within 10 months, 24 out of 178 calluses (which had survived on TDZ-medium from 400 initially plated calluses) developed shoots.

On all media listed in Table 3, a green, compact callus developed (Fig. 1h). Only on TDZ-medium, however, calluses began to form globular structures 2-3 weeks before shoot buds (Fig. 1i) appeared and could be clearly distinguished from non-regenerating calluses.

Generally, once shoot regeneration had started, a prolonged cultivation on TDZ-medium resulted in a permanent proliferation of shoot buds that were incapable of stem elongation and leaf development. Upon transfer to MS-medium supplemented with 0.5 mg·l^{-1} BA, regenerating calluses continued to proliferate shoot buds; however a large portion of these were capable of further shoot development (Fig. 1j). Since all attempts to induce root formation failed, shoots with a length of 0.5-2 cm were grafted onto young seedlings of cv. Mythos (Fig. 1k) and could be transferred into the greenhouse for maturation (Fig. 1l). So far, 21 grafted shoots have developed to plants, of which 15 were fertile (Fig. 1m) and set seed.

Based on these results, further experiments were undertaken in order to test the influence of different TDZ-concentrations with or without NAA upon the viability of calluses and the

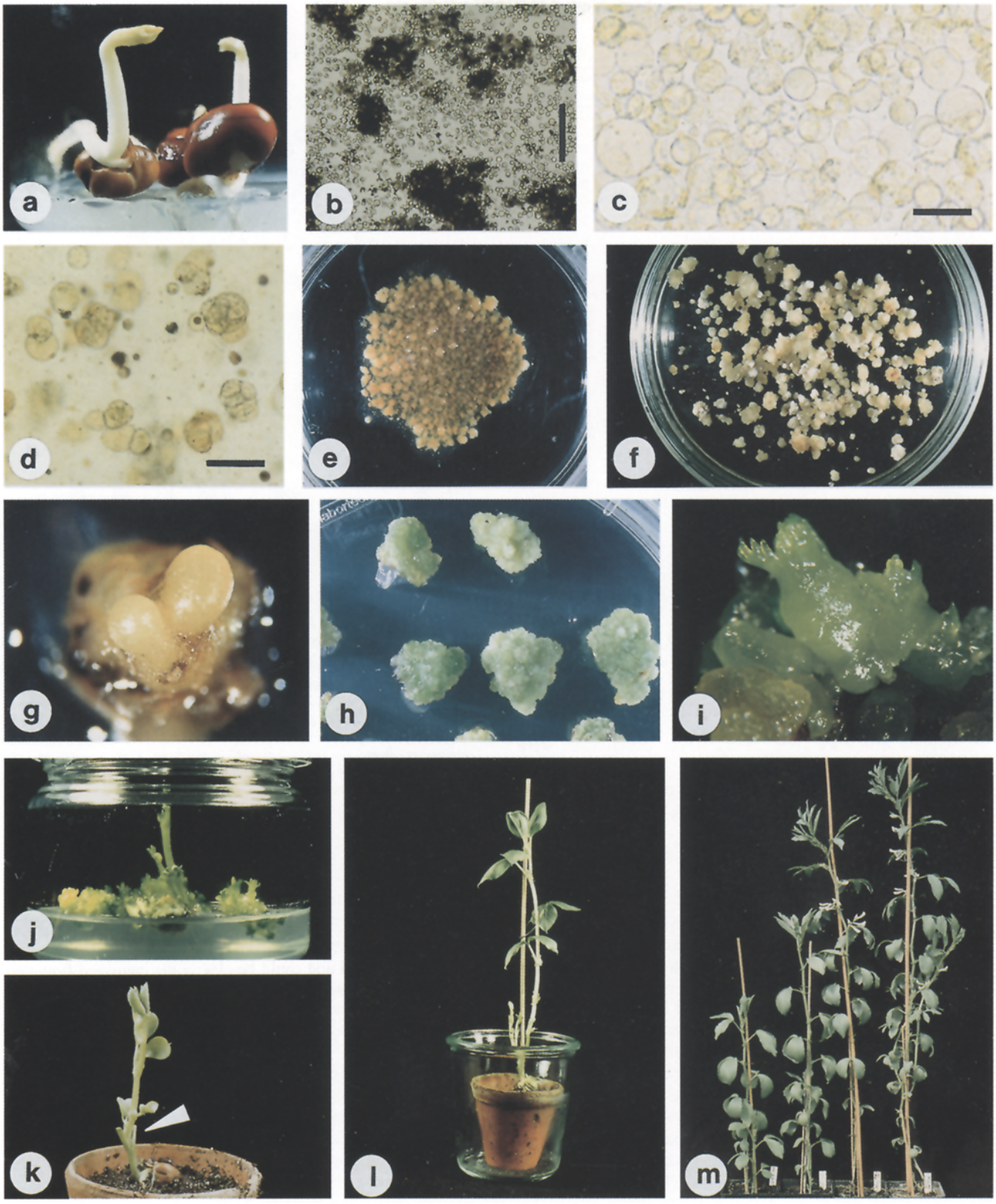

Fig.1. a-m Protoplast culture and plant regeneration in cv. Mythos: (**a**) shoot tips from dark grown seedlings used as protoplast source, (**b**) precipitation of cell debris after treatment with 20 mM $CaCl_2$, (**c**) pure protoplast solution after filtration, (**d**) cell divisions after 15 days in alginate-embedded protoplast cultures, (**e**) lawn of colonies after embedding of protoplasts in alginate type E401, (**f**) protocalluses released from the alginate matrix and transferred to solid medium, (**g**) globular structures arising from 2,4-D- or picloram-induced calluses, (**h**) green, compact callus developing on media supplemented with cytokinins or TDZ, (**i**) shoot-morphogenesis on TDZ-containing medium, (**j**) shoot elongation on medium with 0.5 $mg \cdot l^{-1}$ BA, (**k**) *in vitro*-grafted shoots (attachment site marked with an arrow), (**l + m**) transfer into the greenhouse for maturation. bars=**b** 500 µm, **c** 50 µm, **d** 100 µm

Table 3. Shoot regeneration from protocalluses of *V. faba* cv. Mythos on MS-medium supplemented with different growth regulator-concentrations and -combinations. Experiments were evaluated 10 months after transfer of protoplast-derived calluses to solid medium.

MS-medium supplemented with ($mg \cdot l^{-1}$):	No. of tested calluses*	Calluses with shoot formation
BA: 0.1/0.5/1.0/2.5/5.0/10.0/20.0	250 each	0
BA 0.5, NAA 0.5	207	0
BA 1.0, NAA 0.1	250	0
BA 2.0, NAA 0.75	200	0
BA 5.0, NAA 1.5	200	0
BA 0.5, 2,4-D 0.1	200	0
BA 1.0, 2.4-D 0.5	200	0
BA 2.5, 2.4-D 0.5	200	0
BA 0.5, NAA 0.5, 2.4-D 0.5	200	0
BA 1.0, NAA 0.5, 2.4-D 0.1	150	0
BA 2.5, NAA 1.0, 2.4-D 0.5	150	0
BA 5.0, NAA 2.0, 2.4-D 1.0	100	0
BA 3.0, GA_3 5.0	110	0
BA 0.2, GA_3 0.2, IBA 0.1	150	0
BA 0.4, IBA 0.04	140	0
BA 0.4, IBA 0.5	150	0
BA 0.5, Kin 0.5, 2,4-D 0.5	100	0
BA 0.5, Zea 0.5, Kin 0.5, NAA 0.1	170	0
TDZ 1.0, NAA 0.1	400	24
Zea: 0.1/0.5/1.0/2.5/5.0/10.0	100 each	0
Zea 0.5, 2,4-D 0.5	90	0
Zea 1.0, NAA 0.5	130	0
Zea 2.0, IAA 1.0	80	0
Zea 10.0, ABA 1.0	160	0
Zea 0.5, GA_3 0.5, Dicamba 0.3	100	0
Kin: 0.1/0.5/1.0/2.5/5.0/10.0	100 each	0
Kin 0.5, NAA 0.05	90	0
Kin 1.0, NAA 0.5	100	0
Kin 1.0, 2,4-D 0.5	100	0
Kin 2.5, 2,4-D, 0.75	100	0

*No. of protocalluses which were initially plated on solid media; during subculture over a period of ten months the numbers were reduced by lethal browning of tissue by 80%.

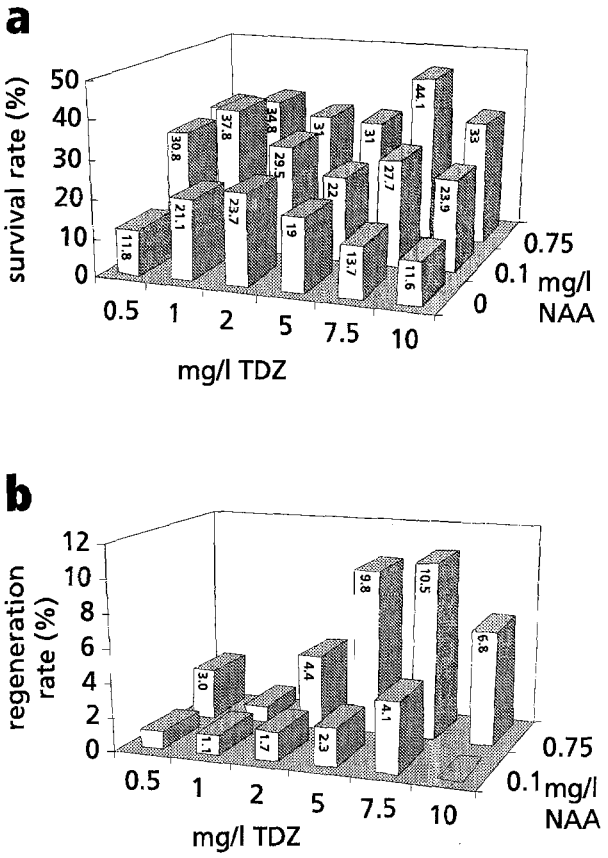

Fig.2. Influence of TDZ- and NAA-concentration on the viability of calluses (Fig. 2a) and the regeneration rate (defined as the percentage of calluses regenerating shoots per no. of surviving calluses, Fig. 2b). The experiments were evaluated six months after transfer of protoplast-derived calluses to solid medium. All conditions were tested simultaneously using protocalluses originating from the same protoplast-isolation (the results of two experiments were combined). A minimum of 100 calluses (survival rate) and 40 calluses (regeneration rate) were tested on each medium.

regeneration efficiency (Fig. 2a+b). These experiments were evaluated after six months of cultivation (this accounts for the lower rate of shoot-producing calluses as compared to the first series of experiments shown in Table 3, which were evaluated after ten months).

All experiments shown in Figure 2 were carried out using protocalluses originating from the same protoplast isolation, making a comparison between different conditions feasible. Approximately 83% of the initial protocalluses turned brown and died on NAA-free medium. In contrast, 28% and 34% of those plated on media supplemented with either 0.1 $mg \cdot l^{-1}$ or 0.75 $mg \cdot l^{-1}$ NAA, respectively, survived, suggesting that NAA had a beneficial effect upon the viability of tissue (Fig. 2a). The regeneration rate (percentage of calluses regenerating shoots per no. of surviving calluses) was positively affected by both TDZ level and NAA concentration, at least up to a concentration of 10 $mg \cdot l^{-1}$ and 0.75 $mg \cdot l^{-1}$ respectively (Fig. 2b).

Taking into account a survival rate of 30% and a regeneration frequency of 5-10% (on MS-medium containing 5-10 $mg \cdot l^{-1}$ TDZ, 0.75 $mg \cdot l^{-1}$ NAA), each alginate disk gives 10-20 shoot-regenerating calluses within 7-8 months. The entire procedure, from protoplast isolation to flowering R0-plants, could be performed in 10-14 months.

Discussion

In this study we report the first successful recovery of plants from protoplast-derived calluses in *Vicia faba*. Regeneration of plants was achieved in only one out of 10 different cultivars tested; however, this low number can not be necessarily attributed to insufficient regeneration capacity. The lethal browning of a considerable high proportion of calluses (up to 100% in some genotypes) due to polyphenolic oxidation led to a low survival rate and hence many experiments prematurely ended. Only cv. Mythos was found to be relatively stable during subculture.

Only alginate-embedded protoplasts cultures were capable of division and prolonged colony formation in KM-medium. Embedding in Seaplaque-agarose or the culture in liquid KM-medium proved unsuccessful. The beneficial effect of sodium alginate embedding has also been described for several other species, e.g. *Arabidopsis thaliana* (Damm and Willmitzer 1988) or apple (Huancaruna Perales and Schieder 1993). Sodium alginate should provide cellular protection against mechanical stress and gradients in environmental conditions during the critical first days of protoplast culture (Draget et al. 1988).

Assuming the existence of different morphogenetic pathways, protocalluses were exposed (i) to auxins to induce somatic embryos and (ii) to cytokinins or thidiazuron alone as well as in combination with auxins to promote shoot development. In spite of the fact that somatic embryogenesis could also be initiated by cytokinins or thidiazuron in some plant species (e.g. Gill and Saxena 1993; Visser et al. 1992), grain legumes generally require auxins in order to stimulate the development of somatic embryos (reviewed by Parrott et al. 1992). In *Vicia narbonensis*, a close relative of the faba bean, embryogenic cultures could be initiated from multicellular explants (Pickardt et al. 1989) as well as from protoplast-derived callus (unpublished) on a medium containing high concentrations of a "strong" auxin (e.g. picloram, dicamba, 2,4-D), followed by a culture step with decreased auxin strength. However, all attempts to induce somatic embryogenesis in protocalluses of *Vicia faba* were unsuccessful.

For shoot organogenesis the cultivation on medium containing thidiazuron was essential, while BA, kinetin or zeatin, alone or in combination with various auxins could not stimulate shoot regeneration. Thidiazuron was originally developed as a cotton defoliant by Schering AG/Germany (Arndt et al. 1976). Several studies have already shown the high potential of thidiazuron for plant regeneration in tissue culture, e.g. in woody plants (see Huetteman and Preece 1993) and also grain legumes (Malik and Saxena 1992). Plant regeneration from protoplasts using thidiazuron as growth regulator was recently reported by Böhmer and Jacobsen (1994) in *Pisum sativum*.

The thidiazuron-induced shoots were incapable of elongation, a phenomenon which has frequently been described e.g. in woody species (see Huetteman and Preece 1993). Since cytokinins generally inhibit shoot elongation, this effect is not unexpected and consistent with the high cytokinin activity of thidiazuron. In *Vicia faba*, insufficient shoot elongation could be overcome simply by exchanging thidiazuron by 0.5 $mg \cdot l^{-1}$ BA, which is known to have lower strength compared to the cytokinin-activity of thidiazuron (Visser et al. 1992; Huetteman and Preece 1993).

Since regenerated shoots were incapable of root formation, shoots were grafted on epicotyls of young seedlings. The technique of grafting described in material and methods is simple and works with 80% efficiency (some experience provided). The same technique has been employed to recover transgenic plants in *Vicia narbonensis* (Saalbach et al. 1994) and plants from protoplast-derived shoots in *Pisum sativum* (Böhmer and Jacobsen 1994).

In our studies we found a beneficial role of NAA in combination with thidiazuron both on viability of calluses as well as on regeneration frequency. Further research is necessary to improve the efficiency of plant regeneration and to extend the procedure to other genotypes. Generally, the establishment of a *de novo* regeneration system is an important step towards the genetic modification at the protoplast level.

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