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Factors affecting protoplast isolation and plant regeneration in *Rosa* spp.

Beeinflussende Faktoren für die Isolierung von Protoplasten und deren Regeneration zu Pflanzen bei *Rosa* spp.

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Introduction

In comparison with conventional breeding methods protoplast cultures offer additional potentials for increasing the genetic variability in cultivated roses. Somatic hybridization may allow the exploitation of a wider genepool, as sexual incompatibilities are bypassed. In addition to nuclear genes also cytoplasmic genes from both parents are combined. By application of special techniques protoplast fusion may even result in new combinations of nuclear and cytoplasmic genomes. Furthermore, isolated protoplasts allow direct genetic transformation with the chance of regenerating non chimaeric transgenic plants. The feasibility of plant regeneration from isolated rose protoplasts was demonstrated for the first time by MATTHEWS et al. (1991) for a *Rosa persica* x *R. xanthina* hybrid genotype. At the Institute for Ornamental Plant Breeding in Ahrensburg integration of protoplast based techniques in breeding of roses is evaluated with special emphasis on possible utilization of new sources of resistance against fungal pathogens such as *Diplocarpon rosae* (blackspot). Prerequisite is the development of a protocol for plant regeneration from protoplasts, which can be efficiently applied to different genotypes. Therefore, factors affecting isolation and regeneration of rose protoplasts were determined.

Material and Methods

Plant Material

Experiments were conducted with rose cultivars ('Pariser Charme', 'Heckenzauber', 'Elina', 'Rouletii'), the hybrid *R.persica x R. xanthina* (obtained from A. Roberts, UK) and selected genotypes of wild species, i.e. *R. canina*, *R. caudata*, *R. corymbifera* 'Laxa', *R. indica*, *R. multiflora* (2 genotypes), *R. spinosissima*, and *R. wichuraiana* (2 genotypes). All genotypes were established as in vitro shoot cultures on MS-media (MURASHIGE and SKOOG 1962) with 30 g/l sucrose and either 1–2 ppm BAP, 0.004 ppm NAA and 0.1 ppm GA_3 or 2 ppm BAP and 0.1 ppm NAA, respectively. Cultures were incubated at 25 °C in a 16-h-photoperiod and subcultured every four to five weeks.

Cell suspension cultures

For initiation of non embryogenic friable callus, leaflets of in vitro grown shoots were injured by transversal cuttings and plated with the abaxial surface on agar solidified MS-media containing 30 g/l sucrose, 1 ppm BAP and either Dicamba (5 and 10 µmol) or Picloram (5 and 10 µmol), respectively. Cultures were incubated at 25 °C in a 16-h-photoperiod and subcultured every four to five weeks on the respective medium. Embryogenic callus of the rose cultivars 'Pariser Charme', 'Heckenzauber' and 'Elina' was induced and maintained according to the method established by DOHM et al. (1996).

Cell suspension cultures were initiated in 200 ml Erlenmeyer flasks using non embryogenic as well as embryogenic callus as starting material. Culture was conducted in MS-media with 30 g/l sucrose, 0.5 ppm 2-iP and 0.5 ppm of either one of the following auxin supplements: 2.4-D, NAA, IAA, CPA, Picloram, Dicamba, IBA and 2.4.5-T. After establishment, cultures were diluted every 14 days with fresh medium in the ratio of 1 : 0.5 or by addition of smaller amounts of medium twice a week. Batch cultures were placed on a gyratory shaker at 90 rpm and incubated at 25 °C in either a 16-h-photoperiod or in continuous darkness.

Protoplast isolation

For the isolation of protoplasts either in vitro shoots, callus, somatic embryos or cell suspension cultures were used as source material. Shoots were chopped in 0.7 M mannitol solution prior to incubation in enzyme solutions. Eight ml of cell suspension in the state of the exponential growth phase were pelleted by centrifugation for 10 minutes at 1000 rpm. The supernatant culture medium was discarded and cell material transferred to 20 ml CPW-solution (cell and protoplast washing medium according to COCKING and PEBERDY 1974) with 1 mg/ml MES (2[N-morpholino] ethanesulfonic acid), 0.6 M mannitol and different combinations and concentrations of the following enzymes: Cellulysin (Calbiochem), Driselase (Sigma), Macerase (Calbiochem) and Pectolyase Y-23 (Kikkoman). En-

zyme incubation was carried out at 25 °C in the dark for 19 hours, followed by one hour of gentle shaking at 50 rpm. The resulting suspension was sieved through a set of gauze with 300, 150 and 100 µm meshsize, respectively. Protoplasts and cell residues were pelleted by centrifugation at 750 rpm. Upon discarding the supernatant enzyme solution, protoplasts were isolated by floating in a CPW-solution with 20 % sucrose. Protoplasts were further washed with CPW-0.6 M mannitol solution and finally with 0.6 M mannitol without CPW-salts. Protoplast yields were determined either by rating or by using a Fuchs-Rosenthal-haemocytometer. In the latter case ten independent samples were counted for each variant and only totally spherical protoplasts with evenly distributed cytoplasma were taken into account. Experiments were performed in a sequence of five repetitions.

Protoplast culture and plant regeneration

Whenever sufficient yield was obtained, protoplast density was adjusted to 100.000 per ml. This suspension was mixed with the same volume of alginate (2.1 % in 0,58 M mannitol). The mixture was pipetted in 0.75 ml aliquots on $CaCl_2$-agar (2.94 g/l $CaCl_2 \cdot 2\,H_2O$, 0,58 M mannitol, 15 g/l agar) in petridishes of 3.5 cm diameter. After one hour, solidified alginate films with immobilized protoplasts were transferred to 1.5 ml liquid culture media in plastic petridishes (Ø 3.5 cm). Protoplast culture media consisted of inorganic salts according to PELLETIER et al. (1983) and organic supplements 8 p according to KAO and MICHAYLUK (1975) with 0.5 M glucose. Media were supplemented with different combinations of 2.4-D, NAA, BAP, 2-iP, Kinetin, Zeatin and TDZ in the range of 0.25 to 3 ppm. Media were adjusted to pH 5.6 and filter sterilized. Cultures were incubated at 25 °C in the dark.

In case of sustained cell divisions the concentration of the osmoticum was reduced by replacing 1/2 of the culture medium every 7 days by a medium with the glucose content decreased by 0.1 M until a final concentration of 0.2 M was reached. When regenerated microcalli had gained a size of approximately 1 mm, alginate films were dissolved in a solution consisting of 20 mM citric acid monohydrat and 0.2 M sucrose (pH 5.6). Calli were subcultured on agar solidified MS-media with various supplements of auxins and cytokinins and incubated at 25 °C in an 16-h-photoperiod. Composition of media, which allowed induction of somatic embryogenesis and further plant regeneration, is summarized in the section results. In a number of experiments, regenerated somatic embryos were transferred to the differentiation medium according to NORIEGA and SÖNDAHL (1991) containing 1 ppm GA_3 and 2 ppm ABA and were incubated at 7 °C in the dark for 4 weeks. Protoplast derived shoots were propagated on MS-media containing 30 g/l sucrose with hormone concentrations adapted to the appearance of the shoots: 0.5 – 2 ppm BAP, 0.004 – 0.1 ppm NAA and 0.1 ppm GA_3. Rooting was achieved upon incubation of elongated shoots on 1/2 concentrated MS-medium with 0.05 ppm IBA, 0.1 ppm NAA and 1 g/l activated charcoal for 2 weeks, followed by another 4 weeks on an MS-medium of 1/4 strength with 0.01 ppm NAA.

Results

Protoplast isolation

In order to find an appropriate procedure for cell wall digestion of rose tissue, a screening of commercially available enzymes was performed with different combinations and concentrations and protoplast release classified by rating according to a five step scale. Cell sus-

Tab. 1. Effectiveness of different enzyme solutions on protoplast release from cell suspensions and in vitro shoots of six rose genotypes
Wirksamkeit verschiedener Enzymlösungen auf die Freisetzung von Protoplasten aus Zellsuspensionskulturen und in vitro Sprossen von sechs Rosengenotypen

Enzyme Composition				Source Material = Cell Suspension Cultures						Source Material = Shoot Cultures					
Cellulysin %	Driselase %	Pectolyase %	Macerase %	Genotype 1	2	3	4	5	6	Genotype 1	2	3	4	5	6
1.5	0.1	0.25	-	+/-	-	++	+/-	-	+/-	-	-	+/-	-	+/-	-
2	0.1	0.25	-	+/-	-	++	-	-	+/-	-	-	+/-	-	+/-	+/-
2.5	0.1	0.25	-	+/-	-	++	-	-	+/-	-	-	+/-	-	+/-	+/-
1.5	0.1	-	0.5	+	+/-	++	+	-	-	+/-	+/-	+/-	+/-	+/-	++
2	0.1	-	0.5	+	+/-	+++	++	-	-	+/-	+/-	+/-	+/-	+++	+++
2.5	0.1	-	0.5	++	+/-	+++	+++	-	-	+/-	+/-	+	+	+++	+++
1.5	0.25	-	0.5	++	+++	+++	++	-	-	+/-	+/-	+	+	+++	+++
2	0.25	-	0.5	+++	+++	+++	++	-	+	+/-	+/-	+	+	+++	+++
2.5	0.25	-	0.5	++	+++	+++	+++	-	+	+/-	+/-	+	+	+++	+++
1.5	0.5	-	0.5	+++	++	+++	+++	-	++	+	+	+	+	+++	+++
2	0.5	-	0.5	++	+	+++	++	-	+/-	+	+/-	+	+	++	+++
2.5	0.5	-	0.5	+	+/-	+++	++	-	+/-	+	+/-	+	+	++	+++
1.5	0.1	0.25	0.5	++	-	+++	+/-	-	+	+/-	-	+/-	+/-	+/-	+/-
2	0.1	0.25	0.5	++	-	+++	+/-	-	+	-	-	+/-	+/-	+/-	+/-
2.5	0.1	0.25	0.5	+/-	-	++	+/-	-	+	-	-	+/-	+/-	+/-	+/-

Genotype 1 = 'Elina'; 2 = *R. indica;* 3 = *R. multiflora* 'Wuci Fenhong'; 4 = *R. corymbifera* 'Laxa'; 5 = 'Rouletii'; 6 = 'Heckenzauber'
Rating of protoplast yield: +++ = very high, ++ = high, + = moderate, +/- = low, - = nil

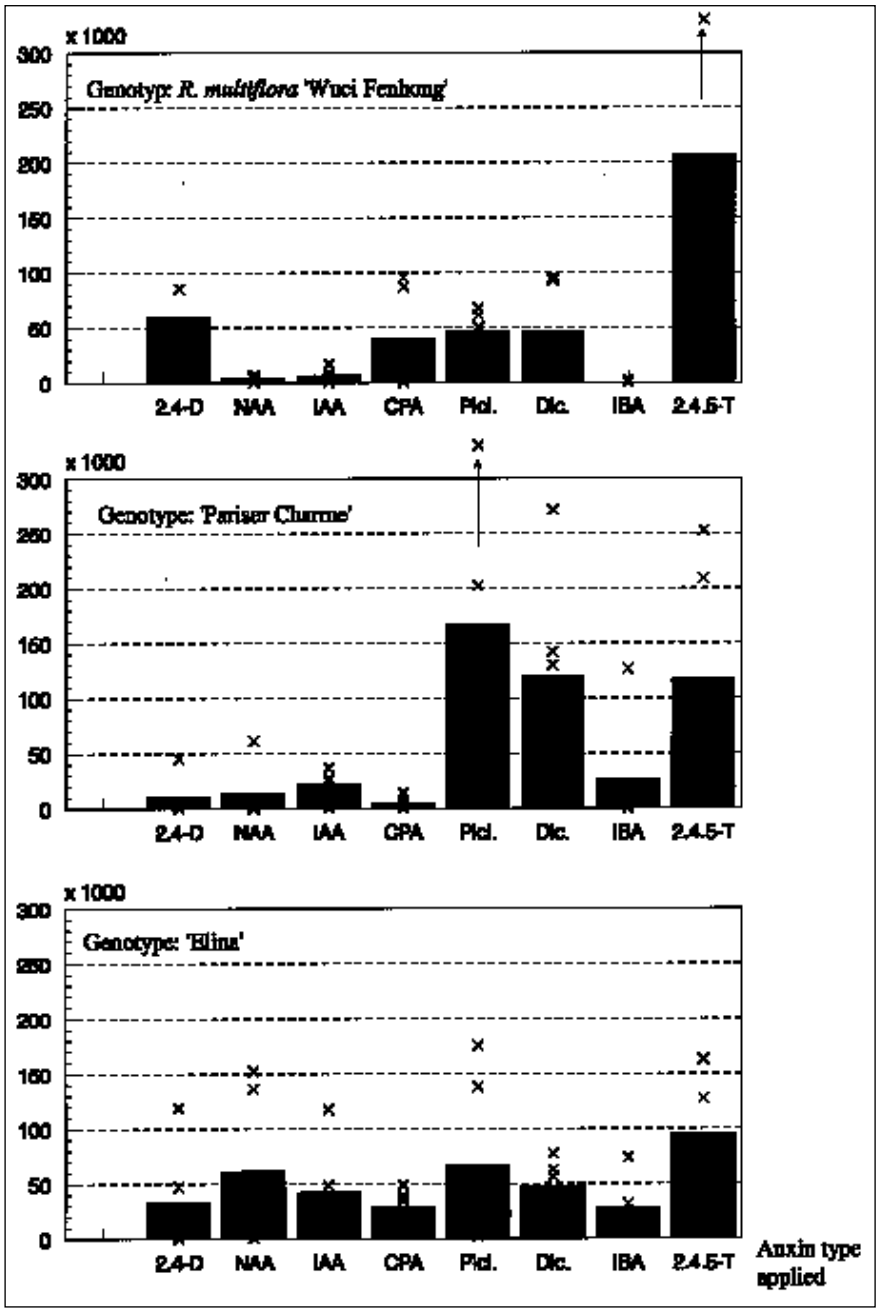


Fig. 1. Protoplast yield per g freshweight obtained from non embryogenic cell suspensions cultured in media supplemented with different types of auxin and incubated in a 16-h-photoperiod (further explanations are found in the text)
Protoplastenausbeuten pro g Frischgewicht aus nicht embryogenen Zellsuspensionskulturen bei Kultur in Nährmedien mit verschiedenen Auxinarten und Inkubation im 16-h-Tag

pension cultures as well as in vitro shoots of six different rose genotypes were compared as protoplast source material. Tab. 1 shows, that protoplast yield varies not only with the composition of the enzyme solution but is highly dependent on the type of source material and the genotype. In case of *R. indica*, *R. multiflora*, *R. corymbifera* 'Laxa' and cv. 'Elina' protoplast release was regularly higher if cell suspensions were used as starting material as compared to in vitro shoots. In contrast, no protoplasts at all could be isolated from cell suspension cultures of cv. 'Rouletii' irrespective of the enzyme concentration and combination. In this case, as well as with cv. 'Heckenzauber', highest protoplast yields were obtained from shoot cultures. A combination of 1.5% Cellulysin, 0.5% Driselase and 0.5% Macerase generally gave best results in isolation of protoplasts from cell suspensions as well as shoot cultures over all rose genotypes and has been used routinely in all further experiments.

As protoplast yield proved to be extremely inconsistent from experiment to experiment, special emphasis was layed on optimization of preculture conditions of source material. The influence of eight different auxins in the culture media for non embryogenic cell suspensions was evaluated for three rose genotypes in terms of protoplast release. Results are shown in Fig. 1, in which the number of isolated protoplasts for each replicate is represented by a cross. The problem of great variation in protoplast yield between different series of experiments is obvious. At the same time, an effect of the kind of auxin in preculture media as well as genotype specific interactions become evident. In case of *R. multiflora* 'Wuci Fenhong' only negligible numbers of protoplasts were set free from cells precultured in media

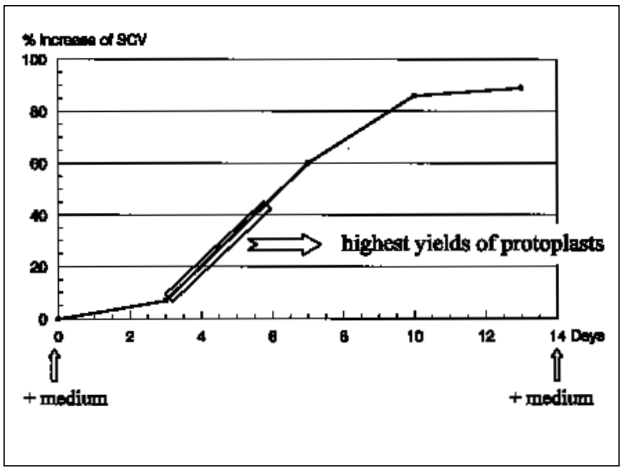


Fig. 2. Growth cycle of rose (cv. 'Elina') cell suspension batch culture, determined by measurement of sedimentated cell volume (SCV)
Wachstumskurve von Suspensionskulturen der Sorte 'Elina', dargestellt anhand der Zunahme des sedimentierten Zellvolumens (SCV)

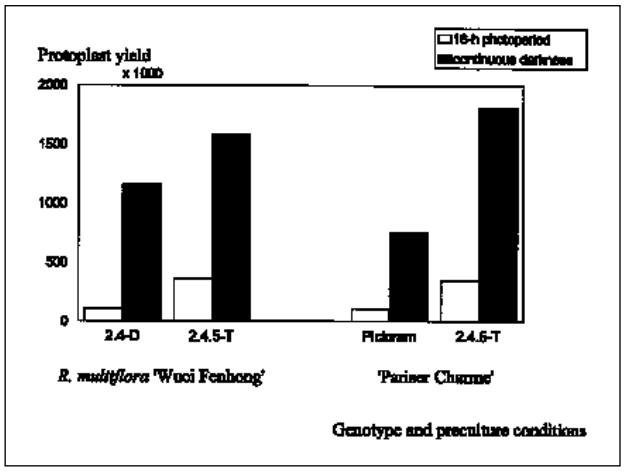


Fig. 3. Protoplast yields per g freshweight from non embryogenic cell suspensions precultured in media with different auxins and under different light regimes
Protoplastenausbeuten pro g Frischgewicht aus nicht embryogenen Zellsuspensionskulturen bei Kultur in Medien mit verschiedenen Auxinen und Inkubation unter unterschiedlichen Lichtverhältnissen

with NAA, IAA or IBA, respectively. Somewhat better results were obtained upon preculture with 2.4-D, CPA, Picloram or Dicamba. However, consistently best results were found when cells used as protoplast source were cultured in presence of 2.4.5-T. In experiments with cv 'Pariser Charme' as well, supplement of 2.4.5-T to the medium led to comparably high protoplast yields. With this genotype similar or better results were obtained upon preculture of cell suspensions in media with Picloram or Dicamba. For the third genotype, 'Elina', protoplast yield was generally low and differences between different preculture media were less pronounced. However, overall most consistent yields were again found after use of 2.4.5-T.

These results were confirmed for embryogenic suspension cultures as well (Data not shown). Preculture of cells in medium containing 2.4.5-T allowed consistent yields for two rose cultivars tested, e.g. 'Heckenzauber' and 'Pariser Charme'. In the latter case, protoplast release was also strongly promoted upon preculture of cells in medium with Picloram in accordance with results obtained from non embryogenic cell material.

Protoplast yield further depends on the appropriate phase within the growth cycle of suspensions. Cell suspensions of roses cultivated as batch cultures and supplemented with fresh culture medium every 14 days, display characteristic growth cycles with lag phase (day 0 – 3), exponential growth phase (day 3 to 10) and stationary phase (day 10–14) as demonstrated in Fig. 2. Protoplast yield proved to be approximately six times higher, when cells within the first three days of the exponential growth phase were used as source material as compared to cells from later stages.

Furthermore, protoplast release was strongly promoted by incubation of cell suspensions in continous darkness as compared to incubation in a 16-h-photoperiod (Fig. 3). The promotive influence of physiologically strong acting auxins as 2.4.5-T or Picloram in preculture media in combination with incubation of source cells in continuous darkness has been confirmed for other genotypes as well, e.g. *R. indica*, *R.canina* and *R. persica* x *R. xanthina*. Protoplast yields in the range of 4 million per gram fresh weight of source cells can be obtained, if the described factors in the preculture of cell suspensions are kept to optimum conditions.

Protoplast regeneration

Protoplasts incubated directly in liquid culture media underwent budding due to incomplete cell wall regeneration, and cell divisions occured only in a few instances. However, upon immobilization in alginate films, sustained cell divisions regularly occured irrespective of genotype, source material and preculture conditions. Initial plating efficiencies of up to 40 % were obtained in culture media supplemented with 0.25 ppm 2.4-D, 1 ppm NAA and 1 ppm of either BAP, 2-iP or TDZ, respectively, as well as in media with 2 ppm NAA and 1 ppm BAP (Fig. 4). Developed microcalli were transferred to agar solidified MS-medium and a range of cytokinin / auxin regimes was tested for induction of morphogenetic processes. With the exception of the hybrid R. persica x R. xanthina, callus derived from protoplasts of non embryogenic origin did not respond to any treatment except for sporadic regeneration of roots. However, protoplasts isolated from embryogenic cell suspension cultures regularly gave rise to an embryogenic type of callus. Somatic embryogenesis occured on auxin containing media (2 ppm NAA plus 1 ppm 2.4-D or 3 ppm 2.4-D according to MATTHEWS et al., 1991) followed by subculture on MS-medium with 1.5 ppm Zeatin, 0.25 ppm NAA and 1 ppm GA_3 (NORIEGA and SÖNDAHL 1991). Somatic embryos occured 9–23 weeks after isolation of protoplasts, depending on genotype and experiment. For further development, the following treatment proved to be most promotive. Embryos of heart stage were transferred to the differentiation medium according to NORIEGA and SÖNDAHL (1991) and incubated at 7 °C in darkness for four weeks. Upon subculture on MS-medium with 100 ppm FeEDDHA, 1 ppm BAP, 0.004 ppm

Tab. 2. Protocol developed for plant regeneration from protoplasts of Rosa spp.
Protokoll zur Pflanzenregeneration aus Protoplasten bei Rosa spp.

Developmental stage	Procedure Steps	Incubation parameter
Isolated protoplasts	Plating in alginate films and culture on 1.5 ml liquid medium with 0.5 M glucose in plastic petri dishes (Ø 3.5 cm)	25 °C darkness
Cell colonies Microcalli	Replacement of 1/2 of the culture medium with medium containing 0.4 M glucose ➔ 0.3 M glucose ➔ 0.2 M glucose in weekly intervals	25 °C darkness
Calli of approximately 1 mm size	Dissolution of alginate films and plating of calli on agar solidified MS-media with 3 ppm 2.4-D	25 °C 16-h-photoperiod
Globular callus	Subculture on MS-medium with 3 ppm 2.4-D	25 °C 16-h-photoperiod
Globular somatic embryos	Transfer to MS-medium with 1.5 ppm Zeatin, 0.25 ppm NAA and 1 ppm GA_3; subcultures as needed	25 °C 16-h-photoperiod
Heart stage somatic embryos	Transfer to Noriega and Söndahl differentiation medium with 1 ppm GA_3 and 2 ppm ABA	7 °C darkness
Heart stage somatic embryos	Transfer to MS-medium with 100 ppm FeEDDHA, 1 ppm BAP, 0.004 ppm NAA and 0.1 ppm GA_3	25 °C 16-h-photoperiod
Somatic embryos with adventitious shoots	Transfer to MS-medium with 1 ppm BAP, 0.004 ppm NAA and 0.1 ppm GA_3	25 °C 16-h-photoperiod
Shoots	Transfer to MS-medium with cytokinin and auxin concentrations adapted to the appearance of the shoots	25 °C 16-h-photoperiod
Elongated shoots	Transfer to 1/2-MS-medium with 0.05 ppm IBA, 0.1 ppm NAA and 1 g/l activated charcoal	25 °C 16-h-photoperiod
Shoots with root primordia	Transfer to 1/4-MS-medium with 0.01 ppm NAA	25 °C 16-h-photoperiod
Rooted plantlets	Transfer to greenhouse	

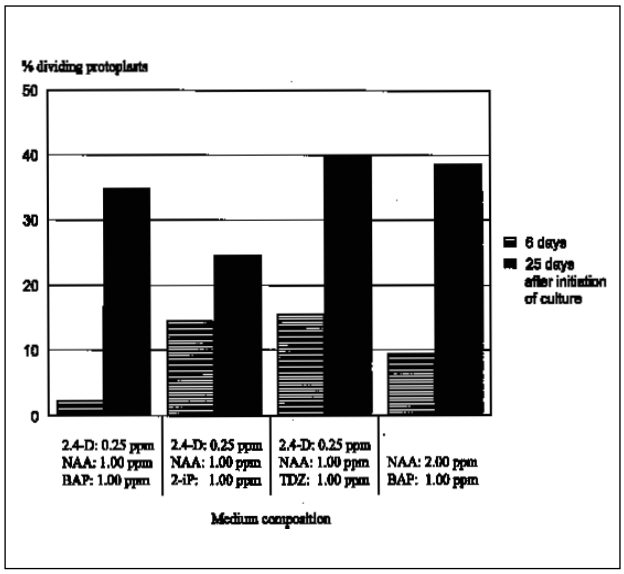


Fig. 4. Colony forming efficiencies of rose protoplasts immobilized in alginate films and cultured in media with different phytohormone supplements
Teilungsraten von Rosenprotoplasten bei Immobilisierung in Alginatfilmen und Kultur in Medien mit verschiedenen Phytohormonzusätzen

NAA and 0.1 ppm GA_3 in a 16-h-photoperiod, part of the embryos turned green and regenerated adventitious shoots. These were cloned, rooted and transferred to the greenhouse. The protocol developed for regeneration of plants from isolated protoplasts in Rosa spec. is summarized in Tab. 2. Different developmental stages are shown in Figs. 5, 6, 7 and 8.

By application of this protocol protoplasts isolated from non embryogenic source material (cell suspensions or in vitro shoots) regenerated callus from 10 genotypes tested so far, i.e. *R. canina*, *Rosa caudata*, *R. corymbifera* 'Laxa', *R. multiflora* (two accessions), *R. spinosissima*, *R. wichuraiana* (two accessions), cv. 'Elina' and cv. 'Pariser Charme'. Protoplasts isolated from embryogenic cell suspensions of cvs. 'Heckenzauber' and 'Pariser Charme', as well as from non embryogenic suspensions of the hybrid *R. persica* x *R. xanthina* were regenerated to plants.

Discussion

A combination of 1.5 % Cellulysin, 0.5 % Driselase and 0.5 % Macerase allows the isolation of protoplasts from in vitro shoot cultures, callus and, preferentially, from non embryogenic as well as embryogenic cell suspension cultures of different rose genotypes. Cell suspen-

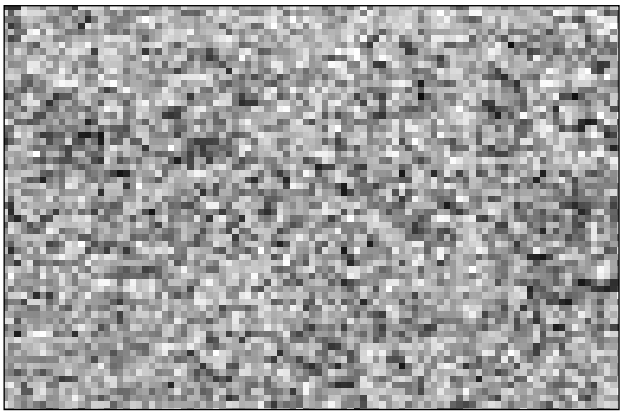


Fig. 5. Freshly isolated rose protoplasts
Frisch isolierte Rosen Protoplasten

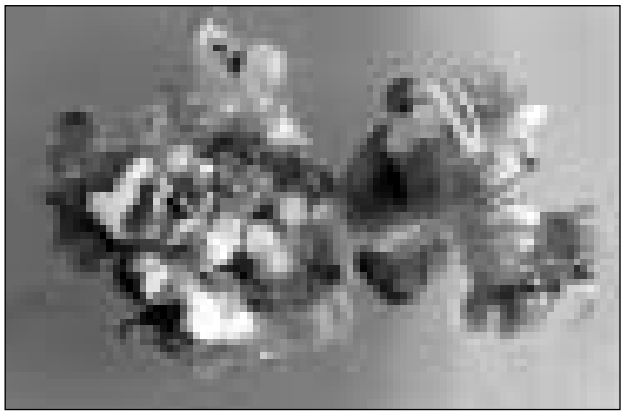


Fig. 7. Regeneration of somatic embryos and adventitious shoots from protoplasts of rose cultivar 'Pariser Charme'
Regeneration von somatischen Embryonen und Adventivsprossen der Rosensorte 'Pariser Charme'



Fig. 6. Regeneration of embryogenic callus from protoplasts of rose cultivar 'Pariser Charme'
Regeneration von embryogenem Kallus aus Protoplasten der Rosensorte 'Pariser Charme'

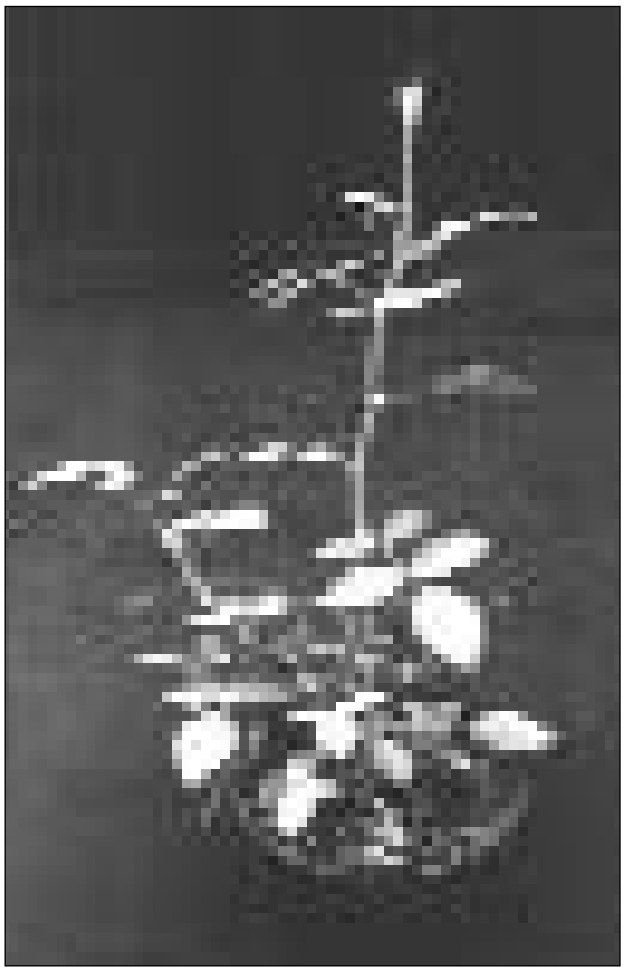


Fig. 8. Plantlet regenerated from protoplasts of rose cultivar 'Heckenzauber'
Aus Protoplasten regenerierte Pflanze der Rosensorte 'Heckenzauber'

sion cultures have been used as source material for protoplast isolation in many crops. Such systems require a callus phase and prolonged proliferation of cells in a dedifferentiated state, thus bearing the potential risk of somaclonal variation. However, with species considered recalcitrant, such as many monocots and woody plants, suspension cultures in many cases offer the advantages of (1) setting protoplasts free more easily,

which (2) possess higher regenerative potentials. Also in *Rosa*, early studies on protoplast culture made use of dedifferentiated cells as source material (PEARCE and COCKING 1973, STRAUSS and POTRYKUS 1980, KRISHNAMURTHY et al. 1979). The first successful protoplast to plant system in *Rosa*, i.e. in the hybrid *R. persica* x *R. xanthina*, comprised the use of embryogenic suspension cultures (MATTHEWS et al. 1991). MARCHANT (1994) compared the suitability of leaves from in vitro shoots, callus and suspension cultures for isolation of rose protoplasts and found that the latter gave the highest yields. MATTHEWS et al. (1994) also consistently obtained highest yields from callus and supension cultures whereas attempts to isolate protoplasts from differentiated tissues such as leaves, stems, petals, flower stalks, sepals, peduncles, and roots were generally unsuccessful. With the exception of cvs. 'Rouletii' and 'Heckenzauber', only negligible numbers of protoplasts could be obtained from in vitro grown shoots, leaflets or somatic embryos in our studies as well. Suspension cultures yielded higher amounts but results were very inconsistent between different experiments unless specific preculture conditions for the source cells were kept to an optimum i.e. (1) supplement of media with physiologically strong acting auxins as 2.4.5-T or Picloram, (2) incubation in continous darkness and (3) use of material at the very beginning of the exponential growth phase. Therefore the routine of supplying the cell suspensions with fresh culture medium every 14 days was changed in later experiments. Addition of smaller amounts of medium twice a week obviously keeps cells in an appropriate state and allows continous isolation of protoplasts.

Protoplast yields in the range of $4 \cdot 10^6$ per gram fresh weight of source cells are obtained if all decisive factors in preculture of cell suspensions are observed. Yields determined in our experiments refer to protoplasts amenable to further manipulations as transformation or fusion procedures. This means that yields were calculated after floating and two further washing steps. Solely totally spherical protoplasts with evenly distributed cytoplasma were taken into account. MATTHEWS et al. (1994) report protoplast yields from rose suspensions in the range of 10^5 per gram fresh weight upon cell wall digestion by a mixture of cellulase and macerozyme. Addition of pectolyase Y23 increased yields to more than $2.5 \cdot 10^7$. MARCHANT (1994) as well found a strong promotive effect of this enzyme in cell wall digestion of rose tissue and discussed its use in protoplast isolation in other members of the *Rosaceae*. In contrast, application of pectolyase Y23 decreased yields drastically in our experiments and proved to be detrimental to protoplast quality and viability. These divergent observations might be explained by inconsistent quality of crude commercial enzyme preparations.

Preculture of source cells in media with physiologically strong acting auxins obviously affects properties of the cell wall in a way to facilitate enzyme digestion. At the same time a surplus of auxin may possibly be detrimental to regeneration capacity of cells. However, embryogenic suspensions did not loose their embryogenic properties during long term incubation of up to two years in media supplemented with 2.4.5-T or Picloram. Embryogenic clusters were observed in liquid culture media, which regularly gave rise to somatic embryos after plating on solidified media. In addition, callus, which was regenerated from protoplasts derived from such embryogenic cell suspensions proved to be of embryogenic nature in every case. Application of the developed protocol allows plant regeneration in two garden rose cultivars, i.e. 'Heckenzauber' and 'Pariser Charme', if embryogenic cell suspensions are used as source material. In case of *Rosa persica* x *R. xanthina*, which is known to have a high regenerative capacity (MATTHEWS et al. 1991), plant regeneration was achieved even though protoplasts had been isolated from non embryogenic cell suspensions.

Plantlets regenerated from protoplasts of *R. persica* x *R. xanthina* did not set flowers yet, but do not show any morphological deviations in vegetative characters. Protoplast derived plants from first experiments with the two cultivars 'Heckenzauber' and 'Pariser Charme', however, exhibited morphological aberrations including an altered plant habitus, i.e. stunted internodes and an extreme outgrowth of axillary buds. The latter might be attributed to an accumulation of cytokinins in the course of in vitro culture, as cuttings taken from the original plants return to the normal growth type. Initial problems with occurance of somaclonal variation might be reduced by the resulting optimized plant regeneration protocol as summarized in Tab. 2, and by frequent replacement of cell suspensions used as protoplast source. Plantlets regenerated in later experiments exhibit less morphological aberrations upon transfer to the greenhouse, but they are still too juvenile for final conclusions. The use of mesophyll derived protoplasts would certainly limit risks of somaclonal variation, as dedifferentiated phases prior to protoplast isolation are avoided. So far, however, culture of such protoplasts in our laboratory as well as in experiments of other groups (MARCHANT et al. 1997; MOTTLEY pers. comm.) resulted only in callus and did not allow plant regeneration.

The protocol for protoplast regeneration in *Rosa spp.* has been developed with the aim of its application to the introgression of resistance genes from wild species by protoplast fusion. A certain degree of somaclonal variation may be tolerable, as resulting somatic hybrids will anyway require further backcrosses with cultivars and strict selection in order to obtain phenotypes of horticultural value. Bases for integration of protoplast techniques in breeding of roses have been set by establishment of procedures for plant regeneration.

Abstract:

A combination of 1.5% Cellulysin, 0.5% Driselase and 0.5% Macerase was found to be most efficient for isolation of protoplasts from in vitro shoot cultures, callus and from non embryogenic as well as embryogenic cell suspension cultures of different rose genotypes. Protoplast yields proved to be highly dependent on the type of source material and the genotype. With the exception of two rose cultivars, use of either shoot or callus cultures or isolated somatic embryos as starting material generally resulted in low protoplast yields. In most cases, however, cell suspension cultures of non embryogenic as well as embryogenic character are preferentially suited for isolation of protoplasts. Specific preculture conditions were found to be essential for obtainig high and consistent release of viable protoplasts

from this kind of material, i.e. (a) the type of auxin in the preculture medium, (b) the appropriate phase within the growth cycle of suspensions, and (c) the light conditions during incubation of source material. Protoplasts incubated in liquid culture media underwent budding due to incomplete cell wall regeneration. However, sustained cell divisions were obtained upon immobilization of protoplasts in alginate films. The protocol developed allows protoplast regeneration in a range of different rose species. Protoplasts isolated from non embryogenic source material gave rise to callus in 10 genotypes, i.e. *R. canina*, *Rosa caudata*, *R. corymbifera* 'Laxa', *R. multiflora* (two accessions), *R. spinosissima*, *R. wichuraiana* (two accessions), as well as in *R.* x *hybrida* cv. 'Elina' and cv. 'Pariser Charme'. Protoplasts isolated from embryogenic cell suspensions of cvs. 'Heckenzauber' and 'Pariser Charme', as well as from non embryogenic suspensions of the hybrid *R. persica* x *R. xanthina* were regenerated into plants.

Zusammenfassung

Ein Protokoll zur Isolierung und Regeneration von Rosenprotoplasten wurde entwickelt, welches sich bei einer Reihe verschiedener Arten und Genotypen anwenden läßt. Von verschiedenen getesteten Enzymlösungen erwies sich die Kombination von 1.5% Cellulysin, 0.5% Driselase und 0.5% Macerase als am besten geeignet für die Isolierung von Protoplasten aus in vitro Sproß-, Kallus- und Zellsuspensionskulturen nicht embryogenen und embryogenen Charakters. Die Protoplastenausbeuten waren in hohem Maße von der Art des Ausgangsmaterials sowie auch vom Genotyp abhängig. Mit der Ausnahme von zwei Rosensorten führte die Verwendung von Sproß- oder Kalluskulturen sowie auch von isolierten somatischen Embryonen generell nur zu geringer Protoplastenfreisetzung. In den meisten Fällen waren dagegen hohe Ausbeuten bei Verwendung von embryogenen oder auch nicht embryogenen Zellsuspensionskulturen zu erzielen. Dabei zeigte sich, daß spezifische Vorkulturbedingungen von entscheidender Bedeutung für hohe und weitgehend konsistente Protoplastenausbeuten aus derartigem Zellmaterial sind. Maximale Ausbeuten im Bereich von vier Millionen pro g Frischgewicht lassen sich erzielen bei Verwendung von Zellen in der frühen exponentiellen Wachstumsphase, Vorkultur in Nährmedien mit physiologisch stark wirkenden Auxinen wie 2.4.5-T oder Picloram und Inkubation des Ausgangsmaterials im Dunkeln. Kultur der Protoplasten direkt in einem flüssigen Nährmedium führte zu starkem „budding" aufgrund einer unvollständigen Zellwandregeneration. Anhaltende Zellteilungen wurden jedoch nach Immobilisierung der Protoplasten in Alginatfilmen erzielt. Protoplasten, welche aus nicht embryogenem Ausgangsmaterial isoliert worden waren, regenerierten bei 10 Genotypen Kallus: *R. canina*, *Rosa caudata*, *R. corymbifera* 'Laxa', *R. multiflora* (zwei Genotypen), *R. spinosissima*, *R. wichuraiana* (zwei Genotypen), sowie bei den Kultursorten 'Elina' und 'Pariser Charme'. Protoplasten, welche von embryogenen Zellsuspensionskulturen von 'Heckenzauber' und 'Pariser Charme' sowie aus nicht embryogenen Suspensionen der Hybride *R. persica* x *R. xanthina* stammten, konnten zu Pflanzen regeneriert werden.

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