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Improved in vitro selection of nitrate reductase-deficient mutants of *Nicotiana plumbaginifolia*

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Summary. The use of increasing knowledge on regulation of nitrate reductase activity in *Nicotiana* cell cultures is the basis for the described optimization of in vitro selection for nitrate reductase-deficient mutants by screening for chlorate resistance. Selection was carried out on haploid mesophyll protoplast-derived cell cultures of *Nicotiana plumbaginifolia*. It is demonstrated that revised selection results in high variant detectability and increased variant confirmability in comparison with the hitherto used selection scheme.

Key words: *Nicotiana plumbaginifolia* – Mutant selection – Nitrate reductase – Protoplast

Introduction

Nitrate reductase (NR) mutants are a tool to study NR, a key enzyme of nitrogen assimilation in higher plants.

Screening of amphihaploid *Nicotiana tabacum* cell cultures for chlorate resistance led to the isolation of the first higher plant mutants that completely lack NR activity (Müller and Grafe 1975, 1978; Müller 1983). The selection method employed was based on the observation that the toxicity of chlorate to plant cells depends on NR activity, presumably because NR catalyses the reduction of chlorate to the highly toxic chlorite (Aberg 1947). To avoid competition between nitrate and chlorate, nitrate-free selection media containing amino acids or ammonium succinate as the sole nitrogen source were usd. This was possible because *N. tabacum* (cv. 'Gatersleben') develops a constitutive NR activity in the absence of nitrate, as shown both for cultured cells (Müller and Grafe 1978) and whole plants (Müller 1983). Moreover, it was found that all *N. tabacum* mutants that lack this constitutive NR activity also lack any nitrate-inducible activity.

The tobacco NR mutants were studied using biochemical and classical genetical as well as in vitro cell genetical methods such as somatic hybridization to study complementation and in vitro selection for detection of revertants (Evola 1983; Glimelius et al. 1978; Grafe and Müller 1982a and 1983, in preparation; Mendel and Müller 1979, 1984; Müller 1983)

A surprising result of the characterization of these mutants was the finding that they belong to only two complementation groups (*nia* and *cnxA*) identical to the main biochemical phenotypes of apoenzyme (*nia*) and cofactor mutants of the molybdenum reparable type (*cnxA*). We tentatively explained this as a result of an interference of the dihaploid state of tobacco with the selection of recessive mutants (Grafe and Müller 1983). In the meantime, in a further selection experiment with tobacco, only cofactor mutants of a type unlike *cnxA* were found. An additional experiment resulted in the confirmation of *nia* and *cnxA* mutants as well as the detection of a further cofactor mutant differing from *cnxA* (Buchanan and Wray 1982).

First attempts to select completely defective NR mutants from monohaploid cell cultures of *N. plumbaginifolia* were successful (Marton et al. 1982). Unsuccessful attempts to regenerate plants of these mutants led to their genetic characterization using complementation in somatic hybrids. It was found that the cofactor mutants represented three complementation groups (Marton et al. 1982). Thus, the better suitability of the monohaploid state for selection of a wide NR mutant spectrum, especially of cofactor mutants, was demonstrated. In subsequent experiments with *N. plumbaginifolia*, regeneration of plants from selected clones completely lacking NR was achieved. Apoenzyme type and two cofactor mutant types could be distinguished (Negrutiu et al. 1983). NR mutants of *N. tabacum* and *N. plumbaginifolia* actually have been shown by means of biochemical methods to fall into four groups: one apoenzyme and three cofactor classes (Mendel et al. 1986). They were demonstrated to be coincidently genetic complementation groups (Xuan et al. 1983).

More cofactor mutant types are necessary in order to obtain more information on the processing of the molybdenum-containing cofactor of NR. Non-reverting mutants (all

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NR mutants so far characterized do revert (Grafe and Müller, in prep.)) would be advantegous for the use of NR mutants for transformation.

Using protoplast-derived cell (p-cell) cultures of *N. plumbaginifolia* unsuccessful attempts to select efficiently NR mutants using procedures similar to those described previously (Marton et al. 1982; Negrutiu et al. 1983) led us to define better conditions for the selection of NR mutants in p-cell cultures.

It was recently observed that ammonium succinate efficiently represses the development of NR activity in *N. tabacum* and *N. plumbaginifolia* p-cells and, that upon depletion of ammonium from the culture medium by proliferating cells, an efficient induction of NR activity was detected even in the absence of nitrate (Marion-Poll et al. 1984). This observation, already described in the literature for some other organisms (Guerrero et al. 1981), was used to develop an improved method of chlorate selection in p-cell cultures of *N. plumbaginifolia*. During these experiments we selected hundreds of chlorate-resistant clones, of which approximately 150 NR mutant plant lines were established. Their further characterization is in progress.

Materials and methods

Plant material

A haploid clone of *N. plumbaginifolia* was used as the protoplast source. The plants were cultured under semi-controlled conditions (dim light and regular nutrition) in a greenhouse as previously described (Chupeau et al. 1974). For a reconstruction experiment we used *in vitro* cultured plants of the confirmed NR-deficient mutant R18 of *N. plumbaginifolia* (Negrutiu et al. 1983).

Protoplast isolation and culture

Protoplasts were isolated from well-expanded leaves (13–15 cm in length) of the immature inflorescences. Protoplasts were cultured in medium T_0 according to Bourgin et al. (1979).

Mutagenic treatment

The mutagenic treatment of two-day old p-cells (i.e., before first cell division) was carried out with ^{60}Co (dose 1,400 rads = 14 J/kg).

Culture of p-colonies for selection of mutants

Five to 6-day old p-cells were transferred to modified medium C (Muller and Caboche 1983) with the initial density of 10^4 dividing p-cells/ml (if not otherwise stated). The medium modifications were substitution of N-salts by 5 mM $(NH_4)_2$ succinate and 10 mM KCl (if not otherwise stated). In some experiments (as indicated in the results) phytohormone concentrations were reduced according to Grafe (in prep.) for improved shoot formation.

Chlorate selection

The chlorate selection was carried out with growing p-colonies cultured in liquid modified medium C, according to three different schemes: A-transfer of p-colonies to selective medium (liquid or solidified with 0.7% agarose and 100 mM chlorate) = classical selection; B-chlorate addition (100 mM) to p-colonies growing in the liquid medium and, after 2 or 3 days after chlorate addition, transfer onto chlorate-free medium; C-Chlorate treatment of growing p-colonies (like procedure B) but transfer after two or three days to chlorate-containing medium (sustained selective pressure).

Confirmation of chlorate resistance

Since chlorate toxicity is expressed on ammonium-containing medium only after depletion of ammonium from the culture medium by growing colonies, the confirmation of chlorate resistance on such a medium was found to be unreliable. Colonies were therefore tested for their inability to use nitrate as the sole nitrogen source assuming chlorate-resistant clones to be deficient for nitrate assimilation. Colonies were cut into two halves, replica plated on modified solid medium C containing either 10 mM KNO_3 or 10 mM $(NH_4)_2$ succinate plus 10 mM KCl, and compared for their ability to grow on these media.

Determination of NR activity

NR activity of *N. plumbaginifolia* cells was measured as previously described for tobacco cells (Marion-Poll et al. 1984).

Results and discussion

Classical chlorate selection

In the first experiments to select for NR mutants in p-cultures of *N. plumbaginifolia* we used the selection schemes described by Marton et al. (1982) and Negrutiu et al. (1983), respectively. This is an adaptation of the scheme (called A in "Material and methods") used already for the first in vitro detection of NR mutants (Müller and Grafe 1978).

We observed (Table 1) that (1) identical procedures gave no reproducible results; (2) survival instead depended on the sample of p-colonies used; (3) especially from cultures derived from mutagenically treated p-cells, numerous surviving colonies were selected, which could not be confirmed as stable variants; (4) quantitatively, selection in liquid media did not differ from selection on agar media; (5) with respect to the qualitative aspects, liquid and agar selection differed markedly: whereas all surviving colonies in the liquid media stayed greenish, many of the agar selected colonies turned brownish, indicating a probable heterogeneity among surviving colonies expressed only on agar media; (6) phytohormone concentrations (0.05–0.5 μM NAA and 1–5 μM BA) had no significant influence (data not shown).

Inadequate usage of the still limited understanding of regulation of NR activity in p-cell cultures was assumed as the possible main reason for this observation. For plant cell cultures, ammonium is known to be an important effector of NR activity. During the initial phase of culture, cells preferentially use ammonium, the assimilation products of which, mainly glutamine, are presumed to repress NR activity. This

Table 1. Surviving colonies after selection A (transfer of p-cells to 100 mM chlorate-containing medium 5 days after protoplast isolation)

Experiment	Mutagenic treatment	Colony survival on			
		Agar media		Liquid media	
		Control	Chlorate	Control	Chlorate
D1	Control	5.2×10^{-1}	6.0×10^{-3}	6.7×10^{-1}	1.0×10^{-4}
	1,400 rad	3.2×10^{-1}	1.0×10^{-1}	3.4×10^{-1}	1.5×10^{-1}
D2	Control	5.0×10^{-1}	9.0×10^{-2}	6.0×10^{-1}	1.2×10^{-1}
	1,400 rad	3.1×10^{-1}	1.0×10^{-1}	3.2×10^{-1}	1.0×10^{-1}
D3	Control	4.5×10^{-1}	1.0×10^{-3}	6.2×10^{-1}	1.2×10^{-3}
	1,400 rad	3.0×10^{-1}	5.0×10^{-2}	2.9×10^{-1}	5.0×10^{-2}

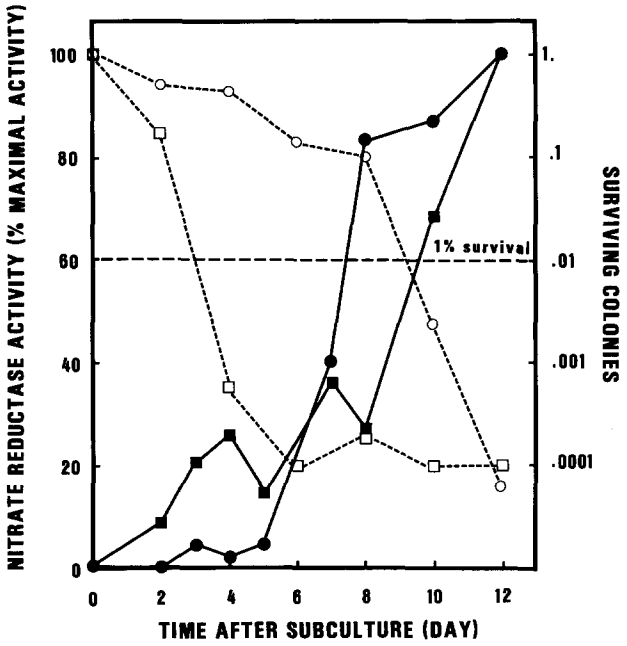

Fig. 1. NR activity and chlorate sensitivity of *N. plumbaginifolia* p-cells cultured in medium C containing ammonium succinate as sole nitrogen source. NR activity is expressed as percentage of maximal induction observed in the cultures 12 days after protoplast-derived cell subculture. Maximal NR activity was 1.7 units (nmol NO_2^-/min per 10^5 plated cells) for cells cultured on medium C containing 1 mM diammonium succinate (■) and 5.8 units for cells cultured on medium C containing 10 mM diammonium succinate (●). Colonies were treated with 100 mM chlorate at various times after their plating at a density of 5×10^3 p-cells/ml in modified medium C containing 1 mM (□) or 10 mM (○) diammonium succinate as the sole nitrogen source. After chlorate treatment for two days, surviving colonies were rescued in chlorate-free medium and scored by eye one month later

repressive effect of assimilated ammonium on NR activity was also confirmed for p-cell cultures of *N. tabacum* (Marion-Poll et al. 1984).

Thus, the classically used transfer of cell colonies for NR mutant selection on an ammonium succinate medium containing chlorate is obviously problematic. Ammonium assimilation during the initial phase of selection represses NR activity and so chlorate can not act selectively on wild type colonies. Instead it can be expected that chlorate during this time expresses the NR-independent effect on cell growth and colony development, respectively, which has been found for NR-deficient mutants in *N. tabacum* (Müller and Grafe 1978). This unfavourable selective situation together with the asynchronous development of p-colonies, especially after mutagenic treatment, may cause the typical picture of an unsharp selection.

Optimization of chlorate selection

Our attempts to improve selection were directed to an adequate usage of the ammonium effect on NR activity. At first we confirmed the time course of NR activity development found with *N. tabacum* (Marion-Poll et al. 1984) for p-cell cultures of *N. plumbaginifolia* (Fig. 1).

Taking this observation into account we added chlorate (50–100 mM) to growing p-cell cultures when nitrate reductase was induced (Fig. 1). Two days later, we transferred the chlorate-treated p-colonies onto non-selective agar medium. The survival curve appears as an almost perfect reflection of the observed NR activity curve. The timing of NR dependent chlorate sensitivity is directly correlated with ammonium supply (Fig. 2).

Assuming the depletion of ammonium from the culture medium to be proportional to biomass production and therefore cell proliferation, a rough estimate of the doubling time of *N. plumbaginifolia* p-cells can be made. This average rate of cell division is approximately 48 h. This means that, under our incubation conditions and in the presence of 10 mM ammonium succinate (Fig. 2), NR would be induced in colonies when they reach a size of 32 to 64 cells per colony. It is very difficult to score cells in colonies of that size but a rough estimate would suggest that the actual average number of cells per colony is higher (100–200 cells per colony). This discrepancy may reflect a variation of biomass per cell when cultures are starving for nitrogen.

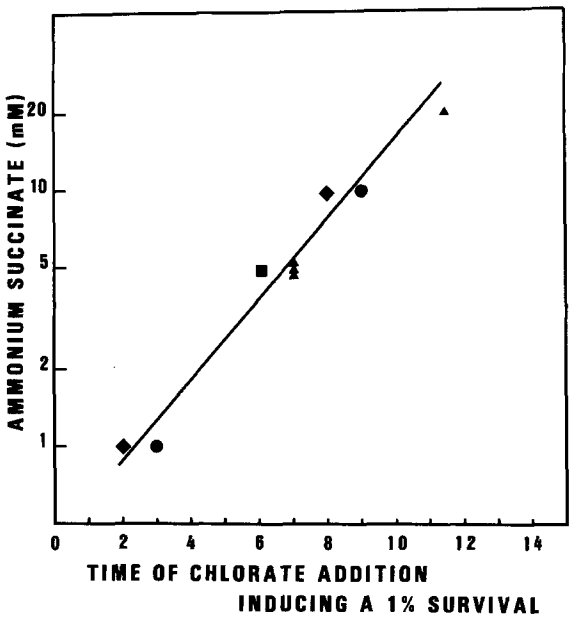

Fig. 2. Chlorate toxicity relative to diammonium succinate concentration in the p-cell culture medium: the curve summarizes survival data observed in different experiments (marked by different symbols for independent experiments). Five days after protoplast isolation (day 0) cells were plated at a density of 5×10^3 p-cells/ml and the timing of chlorate treatments resulting in 1% survival was calculated as shown in Fig. 1

Results of a selection series using this modified selection (selection B) are given in Table 2. Under optimal conditions of chlorate addition in the non-mutagenized cultures of a series of independent experiments colony survival was in the range of 10^{-4}–10^{-5} from which 10% to 100% of these colonies were confirmed to be unable to use nitate. However, this procedure was not efficient to select colonies unable to grow on nitrate from mutagenized cultures.

Revised selection procedure for NR mutants

From the latter observations we decided to combine a 2 day chlorate treatment of positively checked chlorate sensitive p-cultures (directly observed in small samples of the culture) with a sustaining of selective pressure by subsequent transfer to a chlorate-containing agar medium (Table 3).

We included in these experiments the comparison of some parameters of relevance for the selection procedure: initial cell density and ammonium supply. The combination of moderate initial p-cell density (2.5 to 5×10^3 p-cells/ml) with moderate ammonium supply (5–10 mM) provided efficient selective conditions. Increase of initial p-cell density as well as of ammonium supply exerted negative effects on the efficiency of selection.

Table 2. Surviving and confirmed colonies after selection B (short-time chlorate treatment applied directly to growing colonies)

Selection procedure	Time* (days)	Colony survival		Colony confirmation	
		Control	1,400 rad	Control	1,400 rad
B	9 days	3.4×10^{-4}	1.1×10^{-2}	1.1×10^{-5}	2.3×10^{-5}
	10 days	1.2×10^{-5}	8.3×10^{-2}	7.0×10^{-6}	2.1×10^{-5}
	14 days	3.3×10^{-5}	1.2×10^{-2}	n.d.	3.1×10^{-5}
A	9 days	1.3×10^{-3}	7.0×10^{-3}	1.2×10^{-5}	n.d.
	10 days	n.d.	4.0×10^{-2}	n.d.	n.d.
	14 days	n.d.	3.0×10^{-1}	n.d.	n.d.

* Time of chlorate addition to growing colonies for two days before transfer to chlorate-free medium (procedure B) or time of colonies subculture to a chlorate-containing medium (procedure A). Day 0 is p-cell subculture five days after protoplast isolation

Table 3. Surviving and confirmed irradiated colonies after selection C (combination of short-time chlorate treatment and subsequent transfer on solid chlorate-containing media)

P-colony culture			P-colony survival		Variant confirmation	
Density $\times 10^3$/ml	Ammonium (succinate) (mM)	Time* (days)	Total	Frequency	Total	Frequency
10.0	20 (10)	10	600	7.5×10^{-3}	2	2.0×10^{-5}
5.0	10 (5)	10	18	3.9×10^{-5}	14	3.2×10^{-5}
2.5	5 (2.5)	14	3	2.2×10^{-5}	3	2.2×10^{-5}
2.5	20 (10)	14	41	3.0×10^{-4}	8	5.0×10^{-5}

* Calculated from p-cells subculture five days after protoplast isolation

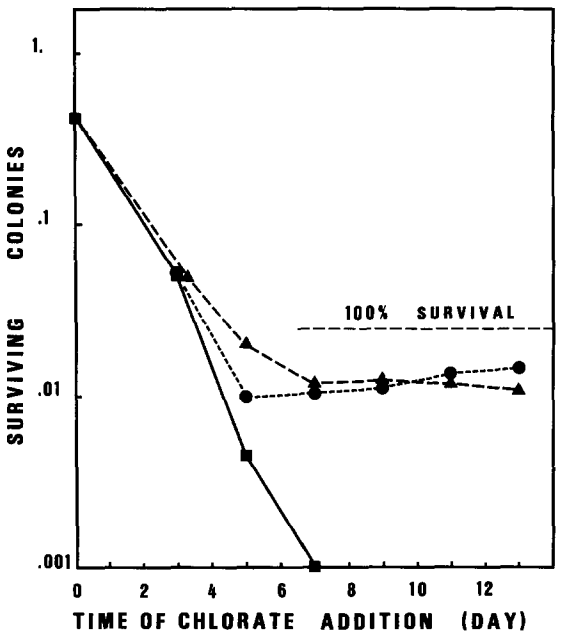

Fig. 3. Recovery of NR-deficient colonies in a reconstruction experiment. P-cell density was 5×10^3/ml. Diammonium succinate concentration was 5 mM. The survival of the wild type p-colony population cultured according to selection procedure B is given as a control (■). Chlorate-resistant colony recovery from a mixed p-colony population initially consisting of 2.5% NR-deficient (R18) p-cells among NR-positive p-cells was performed according to schemes B (●) and C (▲), respectively. The recovery level of 100% is also indicated

We tested the efficiency of this revised selection scheme in a reconstruction experiment. Using a confirmed NR mutant of *N. plumbaginifolia*, R18 (Negrutiu et al. 1983), a mixture initially consisting of 2.5% mutant p-cells in a wild type p-cell population was prepared and selectively cultured according to schemes B and C. Control was a wild type p-cell culture cultured according to scheme B (Fig. 3). From the mixed p-colony population approximately half of the theoretically expected maximal recovery was achieved. Thus, this experiment convincingly demonstrated the efficiency of this mode of selection. These optimized selective conditions were found to be also efficient for the selection of colonies unable to grow on nitrate from mutagenized cultures. In an experiment where protoplasts were irradiated with gamma rays (1,400 rad, survival 65% of controls), the recovery of chlorate-resistant colonies using procedure C was 7.5×10^{-5} among which 84% were confirmed to be unable to utilize nitrate.

The final general selection scheme suggested on the basis of these results involves the following steps: (1) growing of single cell-derived colonies under conditions favourable for optimal induction of NR activity (cell density: 5×10^3 p-cells/ml: ammonium supply: 5 mM diammonium succinate), (2) monitoring of their chlorate sensitivity by adding chlorate to an aliquot of the cell culture, (3) chlorate treatment by adding 100 mM chlorate to positively checked chlorate sensitive colonies (about 15 days after protoplast isolation or 10 days after p-cell subculture under standard conditions), and (4) subsequent transfer on chlorate containing agar media for further culture under sustained selective pressure (10^3 colonies/ml, 100 mM chlorate); (5) confirmation of the inability to grow on nitrate of the selected colonies.

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