Plant Physiol. (1988) 86, 266–269
0032-0889/88/86/0266/04/$01.00/0

Allyl Alcohol Selection for Lower Alcohol Dehydrogenase Activity in *Nicotiana plumbaginifolia* Cultured Cells[1]

Received for publication May 8, 1987 and in revised form September 23, 1987

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ABSTRACT

One cell strain with stable tolerance to allyl alcohol (AA^r) was selected from 6 × 10^8 suspension cultured *Nicotiana plumbaginifolia* Viviani cells. The selected strain contained one-half the alcohol dehydrogenase (ADH) activity of the wild type (NP) due to the loss of two of three bands of ADH activity seen on starch gels following electrophoresis of wild-type cell extracts. Anaerobic conditions, simulated by not shaking the suspension cultures, increased the ADH specific activity to more than 3-fold the initial level in both strains but did not change the number of activity bands or the relative levels of activity. The cell strain with decreased ADH activity lost viability more rapidly than the wild type under the anaerobic conditions. The AA^r cells were 10 times more tolerant to ethanol than the NP cells and were also somewhat more tolerant to acetaldehyde and antimycin A. The substrate specificities of the ADH enzymes from both strains were very similar. Further selection of AA^r cells with allyl alcohol produced strains with even lower ADH activity and selection under anaerobic conditions produced strains with increased ADH activity. Genetic studies indicate that the *N. plumbaginifolia* ADH activity bands arise from subunits produced by two nonallelic genes. This is the first example of the use of allyl alcohol to select for decreased ADH using cultured plant cells.

Alcohol dehydrogenase (ADH)[3] (EC 1.1.1.1.) catalyzes the reversible interconversion of ethanol and acetaldehyde with the cofactors NAD and NADH, respectively. The plant enzymes are dimeric with subunits of approximately 40 kD size (5, 7) and can utilize many primary and secondary alcohols as substrates.

In plants ADH and a series of other proteins are induced by anaerobic conditions such as flooding (14, 15). The ADH activity would appear to have an important function in this stress response since germinating seeds of *Zea mays* (16) and *Hordeum vulgare* (8) mutants deficient in ADH activity (lack Adh1 activity) are very sensitive to flooding and die sooner than the wild type under such conditions.

The Adh1 null mutants of maize can be selected by treating pollen with allyl alcohol (18) which is converted by ADH to the more toxic compound acrolein. Thus pollen grains containing ADH activity can be killed and those lacking ADH will remain viable and be capable of carrying out pollination. Such a selection system has been used to select yeast with decreased levels of ADH (11) and animal cells with from 15 to 40% of the normal ADH activity (20). However no successful selections for allyl alcohol resistance have been reported with cultured plant cells.

In this report we describe the selection of an allyl alcohol tolerant *Nicotiana plumbaginifolia* strain with decreased ADH activity and present some physiological and enzymatic characteristics.

MATERIALS AND METHODS

Cultures and Culture Conditions. Callus cultures of *Nicotiana plumbaginifolia* were initiated from surface-sterilized seeds (10% clorox for 10–15 min and rinsed twice in sterile water and soaked for 1 h in 0.5 mg/ml GA_3) germinated on agar-solidified (0.8%) Murashige and Skoog medium (12) supplemented with 3% sucrose and 0.4 mg/L 2,4-D. Suspension cultures were grown in 125 ml Erlenmeyer flasks containing 50 ml of the same medium without agar on a gyratory shaker at 130 rpm at 27 ± 1°C under continuous fluorescent illumination at 1000 lux. The suspension cultures were subcultured every 7 d by inoculating 5 ml of suspension into 50 ml of fresh medium.

Selection and growth studies were initiated by inoculating 1.0 or 0.5 g fresh weight cells, respectively, into 50 ml liquid medium containing the test compound—followed by incubation on a gyratory shaker for the times specified. Allyl alcohol, ethanol, and acetaldehyde were added to the culture medium without sterilization while antimycin A was added in 10 μl ethanol to each 50 ml of liquid medium. Cell fresh weights were determined by collecting the cells on Miracloth under vacuum and then weighing the collected cells. Cell viability was measured by examining 100 cells from each flask for dye exclusion using phenosafranin (21).

ADH Assay *in Vitro* and in Starch Gels. Cells collected as above were homogenized in a glass-Teflon homogenizer with a ratio of 1 g fresh weight cells to 2 ml grinding buffer (10 mM Tris [pH 8.0], 2 mM DTT, 1% [w/v] polyvinylpolypyrrolidone). The homogenate was centrifuged at 25,000*g* for 20 min at 4°C and the supernatant was used for the ADH assay or starch gel electrophoresis.

The ADH activity was usually measured with ethanol as substrate by measuring NADH production from NAD by increase in *A* at 340 nm at 25°C in a double beam recording spectrophotometer by a modification of the method of Efron and Schwartz (6). The reaction mixture contained 750 μmol Tris-HCl (pH 9.0), 3 μmol NAD, and 1% (v/v) ethanol in a final volume of 5.0 ml. The reaction was initiated by adding ethanol and the *A* changes noted before this addition were subtracted from the ethanol induced rate. Protein was estimated by the method of Bradford (1) using BSA as standard.

Starch gel electrophoresis and activity staining of ADH isozymes were performed according to Brewbaker *et al.* (2) and Brewer (3) with minor modifications. Horizontal starch gels were prepared with 13% Connaught electro-starch in 5 mM histidine

[1] Funding from the Illinois Agricultural Experiment Station and the National Science Foundation grant 79-13956.

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[3] Abbreviations: AA^r, allyl alcohol resistant cell line; ADH, alcohol dehydrogenase; NP, wild-type *Nicotiana plumbaginifolia* cell line.

buffer (pH 8.0) as gel buffer and 0.41 M citrate buffer (pH 8.0) as bridge buffer. Gels were run at constant current (20 mA) for 4 h in a refrigerator (0–5°C). ADH activity was stained by incubation of the cut surface of the starch gel in a solution of 50 mM sodium phosphate buffer (pH 7.0), 0.4 mM nitroblue tetrazolium, 0.15 mM phenazine methosulfate, 1 mM NAD, 7% (v/v) ethanol at 25°C in the dark. Bands of ADH activity became visible within 30 min and development was allowed to progress until the bands had reached the desired intensity.

RESULTS AND DISCUSSION

Allyl Alcohol Selection. Growth studies with suspension cultures of the wild type NP cells showed that 18 μM allyl alcohol or higher completely inhibited growth (Fig. 1). Selection for resistance was carried out by incubating the NP cells in 20 μM allyl alcohol. After 3 to 4 months cell growth was seen in 16 of the 40 flasks inoculated, but cells from only one of these flasks grew further when placed back into 20 μM allyl alcohol. If we assume that this one tolerant strain originated from a single cell then the tolerance frequency was approximately 1.7×10^{-9} based on cell counts of 1.5×10^{7} cells/g fresh weight determined previously with this same cell line (9).

The selected strain denoted AA^r, could tolerate higher levels of allyl alcohol than the NP cells with an I_{50} value of 32 μM in comparison to an I_{50} value for the NP cells of 11.5 μM (Fig. 1).

Initial ADH activity measurements in crude extracts showed that the AA^r cells had about one-half as much activity as the NP cells. This change was due to the loss from the AA^r cells of two of the three ADH isozyme bands normally detected following starch gel electrophoresis of NP cell extracts (Fig. 2). The NP cells show three anodal activity bands but the AA^r cells lack the slower moving two bands. The loss of expression of the two bonds was stable over at least a 1 year period since an identical pattern was seen in AA^r cells grown under nonselective conditions during this time period.

The loss of two of the three ADH isozyme bands originally present in the parental NP cells upon allyl alcohol selection indicates that activity from one ADH structural gene has apparently been lost. The active ADH enzyme is usually composed of dimers which can come from alleles at the same locus or from separate gene loci (17). Thus the simplest model to explain the changes in isozyme patterns (Fig. 2) is that the NP cells have two ADH genes (allelic or nonallelic) producing subunits with electrophoretic mobilities different from each other. These form homodimers which make up the two outside bands while the band in between these is made up of heterodimers. If one of the original ADH genes becomes inactive for some reason then the homodimer as well as the heterodimer bands would be lost as seen in the AA^r cells. This loss also correlates with the decreased total enzyme activity found in AA^r cells.

The subunits making up the ADH molecules in the NP cells could be produced by two allelic genes or by two nonallelic genes. To determine which possibility is correct ADH isozyme banding

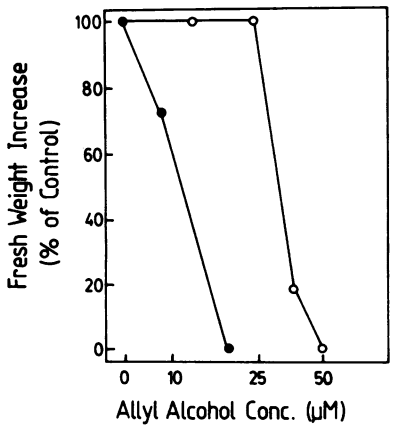

FIG. 1. Effect of allyl alcohol on the growth of AA^r (○) and NP (●) cells grown in liquid medium. One g fresh weight cells were incubated for 7 d with duplicate flasks for each concentration. The control fresh weights were 11.4 for AA^r and 12.6 for NP.

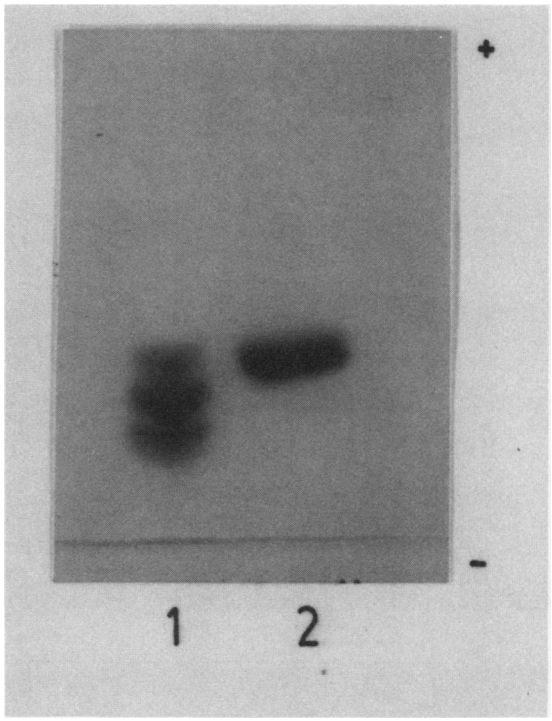

FIG. 2. Electrophoretic analysis of ADH isozymes in wild-type (NP, lane 1) and ADH deficient (AA^r, lane 2) *N. plumbaginifolia* cells. Equal amounts of protein were loaded in each lane.

Table I. *Induction of ADH Activity and Decrease in Viability and Fresh Weight of AA^r and NP Cells Incubated in Liquid Medium without Shaking*

Time after Inoculation	NP Cells		
	ADH activity	Fresh weight	Viability
d	*μmol min⁻¹ mg⁻¹ protein*	*g/2 flasks*	*%*
0	0.81 ± 0.14[a]		85 ± 2[c]
1	1.64 ± 0.09	1.76 ± 0.45[b]	78 ± 7
2	1.88 ± 0.61	1.74 ± 0.25	77 ± 8
3	1.86 ± 0.02	1.74 ± 0.25	70 ± 8
4	1.85 ± 0.12	1.62 ± 0.30	68 ± 7
5	2.56 ± 0.16	1.44 ± 0.12	54 ± 8
	AA^r Cells		
0	0.42 ± 0.09		83 ± 2
1	0.62 ± 0.04	1.24 ± 0.24	69 ± 5
2	0.86 ± 0.06	1.17 ± 0.21	36 ± 16
3	1.16 ± 0.11	1.14 ± 0.12	30 ± 7
4	1.14 ± 0.07	1.19 ± 0.14	22 ± 16
5	1.64 ± 0.16	1.18 ± 0.15	24 ± 16

[a] Mean of two experiments ± standard deviation. [b] Mean of two experiments ± standard deviation with the initial inoculum being 2 g fresh weight total in two flasks each containing 50 ml liquid medium. [c] Mean of two flasks each in two experiments ± standard deviation measured by phenosafranin dye exclusion (21). The differences between the means on d 2 to 5 are significant at the 95% level by the *t* test.

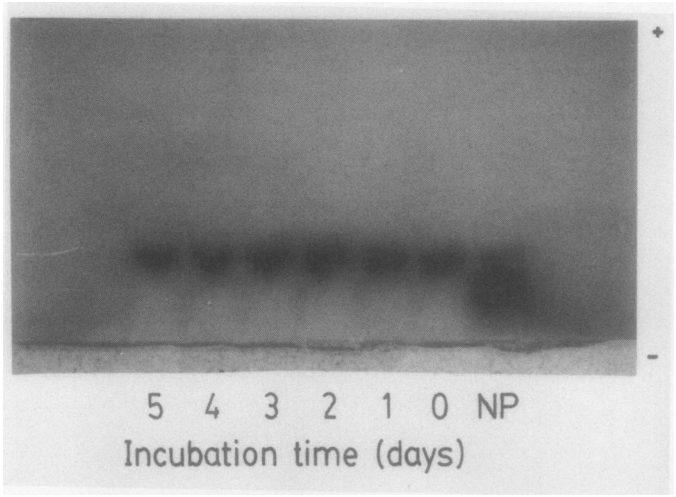

FIG. 3. Effect of anaerobic conditions on the ADH isozyme pattern of AA^r cells. AA^r cells were incubated in unshaken liquid medium as in Table 1 for the times listed. Extract from uninduced NP cells was included as a control. Equal amounts of protein were loaded in each lane.

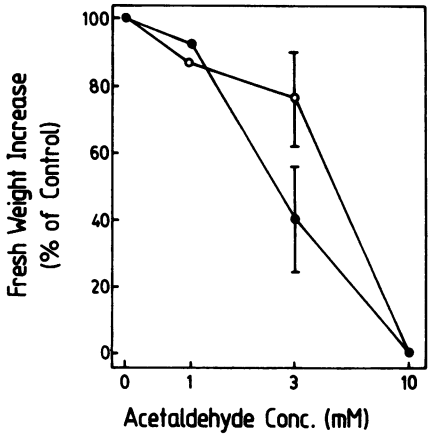

FIG. 5. Effect of acetaldehyde on the growth of AA^r (O) and NP (●) cells grown in liquid medium. One-half g fresh weight cells were incubated for 14 and 10 d, respectively, with 7 flasks at each concentration. The mean control fresh weights were 9.9 and 7.8 g, respectively, with *bars* on the figure representing the standard deviation. The means are significantly different at the 3 mM concentration at the 95% confidence level by *t* test.

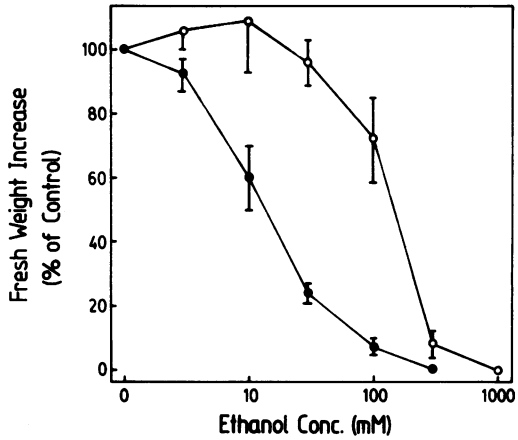

FIG. 4. Effect of ethanol on the growth of AA^r (O) and NP (●) cells grown in liquid medium. One-half g fresh weight cells were incubated for 14 and 10 d, respectively, with four flasks at each concentration. The mean control fresh weights were 6.7 and 10.8 g, respectively, with *bars* on the figure representing the standard deviation.

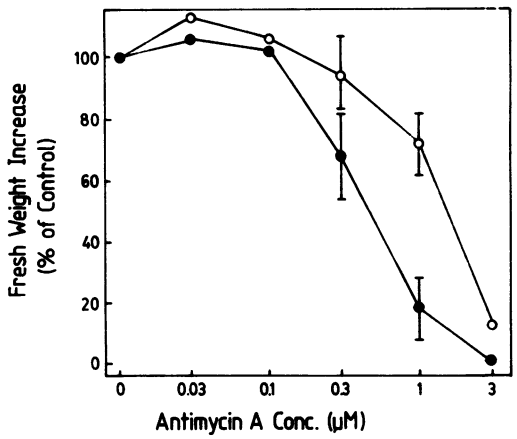

FIG. 6. Effect of antimycin A on the growth of AA^r (O) and NP (●) cells grown in liquid medium. One-half g fresh weight cells were incubated for 14 and 10 d, respectively, with four flasks at each concentration. The mean control fresh weights were 6.9 and 8.6 g with no additions and 7.6 and 8.6 with 10 μl ethanol added for the respective cell strains. *Bars* on the figure represent the standard deviation. The means of the 1 μM concentration are significantly different at the 95% confidence level by *t* test.

patterns were determined using extracts from suspension cultures initiated from one *N. plumbaginifolia* plant and from 28 progeny produced by self-fertilization of this plant. In all cases the three banded pattern was seen. This indicates that the ADH subunits in the parental plant were formed by two nonallelic genes since no segregation was seen in 28 selfed progeny. If the ADH subunit genes were allelic then one-fourth of the progeny would be homozygous for one of the genes and have only one band, one-fourth would be homozygous for the other gene and have only the other band and one-half would still be heterozygous and have all three bands like the parent plant. Independent genetic evidence for the nonallelic nature of the *N. plumbaginifolia* ADH genes has also been found by I. Negrutiu (personal communication).

Effect of Anaerobic Conditions on ADH Activity and Cell Viability. When the NP and AA^r cells were inoculated into fresh liquid medium and incubated without shaking to produce anaerobic conditions, the ADH levels increased greatly within 24 h and by 5 d the levels were more than three times the initial levels (Table I). The anaerobic induction conditions did not change the isozyme banding pattern of the AA^r cells, however, and only one band was seen in cell samples taken daily during the 5 d experiment (Fig. 3). The ADH specific activity levels in both NP and AA^r cells incubated for 5 d under normal shaking conditions declined by about 30 to 60%.

The AA^r cells lost viability more rapidly than the NP cells under the anaerobic conditions with a 50% loss occurring by d 2 with the AA^r cells and a 50% loss occurring after d 5 for the NP cells (Table I). While the AA^r cells lost viability at a rapid rate, the level of cell death appears to plateau by d 4 and 5 with about 20% viable cells. Likewise the AA^r cells in a similar experiment carried out for 8 d reached a viability level of 20 to 25% and were still capable of growing when replaced on the shaker. The fresh weights of the AA^r cells in the unshaken flasks also declined more rapidly than those of the NP cells (Table I). The measured decline in fresh weights is apparently correlated with the decline in viability as dead cells often disintegrate and lose their contents so would weigh less.

The increased sensitivity to hypoxia is similar to that seen with

Table II. *Substrate Specificity of the ADH Reaction in Crude Extracts from NP and AA^r cells*

The forward reaction contained 1.0% alcohol (v:v) and 600 μM NAD while the reverse reaction contained 0.5% acetaldehyde and 120 μM NADH. Activity was measured spectrophotometrically at 25°C in two separate experiments with two measurements in each experiment with AA^r and NP cell extracts with ADH activities with ethanol of 0.52 and 1.52 or 1.17 and 2.67 μmol min^{-1} mg^{-1} protein, respectively.

Substrate	Forward Reaction	
	NP cell extract	AA^r cell extract
	% of control activity	
Ethanol	100	100
Allyl alcohol	95 ± 1	88 ± 13
n-Butanol	81 ± 4	74 ± 1
n-Propanol	56 ± 1	46 ± 1
Isoamyl alcohol	38 ± 4	31 ± 3
Isobutanol	12 ± 0	10 ± 2
Isopropanol	0	0
Methanol	0	0
	Reverse Reaction	
Acetaldehyde	188 ± 6	180 ± 29

the ADH nulls of maize and barley (8, 16) which also are more sensitive to such conditions. The anaerobic toxicity to low ADH mutants is proposed to be caused by increased lactic acid formation in lieu of ethanol formation, thus causing cytoplasmic acidification (13).

Tolerance to Ethanol, Acetaldehyde, and Antimycin A. When the ethanol tolerance of the AA^r and NP cells was tested the AA^r cells were clearly more tolerant with an I_{50} concentration of 148 mM while the NP I_{50} concentration was 13.5 mM (Fig. 4). The AA^r cells were also somewhat more tolerant to acetaldehyde than the NP cells with an I_{50} concentration of 4.6 mM *versus* 2.5 for the NP cells (Fig. 5). Dhaliwal and King (4), however, reported that Adh1$^-$ *Zea mays* callus cultures were much more sensitive to acetaldehyde than were the wild type.

The AA^r cells were also slightly more tolerant to antimycin A (I_{50} = 1.5 μM) than the NP cells (I_{50} = 0.47 μM) (Fig. 6). These results contrast with those of Shimamoto and King (19) who showed that the respiratory inhibitor, antimycin A, lowered the plating efficiency of ADH-deficient *Z. mays* cells more than that of the wild type. The Adh1-0 mutant was used in these experiments which typically showed 7 to 8% of the wild-type ADH activity when in culture (19).

Characteristics of the ADH Enzyme Activity in Cell Extracts. The ADH activity from the NP and AA^r cells had very similar substrate specificities ranging from the highest to lowest utilization rate: ethanol, allyl alcohol, *n*-butanol, *n*-propanol, isoamyl alcohol, isobutanol (Table II). Isopropanol and methanol did not serve as substrates. The reverse reaction utilizing acetaldehyde as substrate was almost double the reaction rate with ethanol as substrate with ADH from both cell lines.

These substrate utilization rates are similar to those found with ADH from pea, bean and lentil where *n*-butanol and *n*-propanol were good substrates while methanol and isopropanol were poor (10). Likewise *Arabidopsis* ADH utilized allyl alcohol and n-butanol well and methanol, isobutanol and isopropanol poorly (5). However, *Arabidopsis* ADH used isoamyl alcohol and *n*-propanol very poorly in contrast to the *N. plumbaginifolia* ADH shown here (Table II).

Selection for Lower and Higher ADH Activity. Additional selection was carried out with the AA^r suspensions inoculated into 15 flasks each of 200 and 400 μM allyl alcohol. The cells grew up within 3 months in only 5 of the 200 μM allyl alcohol flasks. Four of these five selected strains continued to grow when reinoculated into 100 μM allyl alcohol. After four transfers in 100 μM allyl alcohol medium, the ADH activity of the four cultures was determined to be 0.077, 0.062, 0.110 and 0.167 μmol min^{-1}mg^{-1} protein in comparison to 0.16 for AA^r and 0.75 for NP cells at the same growth stage (early log phase). The ADH levels in the same four cultures after four more transfers in medium without allyl alcohol were 0.084, 0.053, 0.122, and 0.138 μmol min^{-1}mg^{-1} protein, respectively. Thus it would appear that allyl alcohol selection can also select for further decreases in ADH activity and that these low levels are stable.

Several flasks used in the anaerobic induction experiment of Table I were left sitting for 22 d without shaking and clumps of cells began to grow in the stationary flasks. Four of these clumps were removed and were cultured under normal shaking conditions with biweekly subculture for three months. The ADH activity of these four strains was found to be 0.16, 0.33, 0.46, and 0.65 μmol min^{-1}mg^{-1} protein in comparison to an activity of 0.16 for the original AA^r cells at the same growth stage. The ADH levels of these strains after four more transfers without additional selection were 0.24, 0.33, 0.28 and 0.37 μmol min^{-1}mg^{-1} protein, respectively. Thus it seems that it is also possible to use anaerobic conditions to reselect variants which retain near wild type ADH activity levels. These cultures still contain only one ADH activity band following electrophoresis so the increased activity would appear to not be due to selection of contaminating wild type cells or to revertants.

Acknowledgments—This work was carried out with the technical assistance of Joan Hoppenworth and Robert Finke.

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