Aluminum Tolerance in Alfalfa as Expressed in Tissue Culture

W. A. Parrot and J. H. Bouton*

ABSTRACT

Acid soils whose pH is low enough for Al to become soluble are found throughout the world and greatly limit growth of Al-sensitive crops like alfalfa, *Medicago sativa* L. Alfalfa germplasms were selected to overcome these conditions and are now available for testing. The objective of this study was to test performance of an acid-tolerant germplasm (AT), and an acid-sensitive check germplasm (AS), in acid, Al-toxic cell culture conditions. Both germplasms were tested for growth as callus in normal Blaydes medium, a Blaydes medium modified by lowering pH and levels of Ca and P, and on the same modified medium but with a toxic Al level. The modified Blaydes medium depressed callus growth of both germplasms relative to their growth on normal Blaydes. This depression was equal for both germplasms of the first 6 wk of culture, but by the 8th wk, the AT germplasm outperformed the AS germplasm. The addition of Al further decreased growth of AS callus but not AT callus. This suggests that plants from the AT germplasm rapidly express Al tolerance at the tissue level. With increasing time in culture, the AT germplasm also expresses acid tolerance in culture. The differences in callus growth shown by genotypes within germplasms indicates the cell culture procedure can be used to screen genotypes for acid or Al tolerance. Significant genotypic variability for acid, Al tolerance is still present within the germplasm, suggesting that continued progress from selection should still be possible.

ACID SOILS are a severe impediment to the growth of alfalfa in many areas of the world. This is especially true if the soil contains toxic levels of Al and Mn, two metals that become soluble, and therefore accessible to plants, at low pH values. The extent and severity of these soil problems, coupled with alfalfa's sensitivity to these conditions, have led researchers to propose using whole plant selection in a breeding program to increase tolerance to acid soils (Devine et al., 1976; Bouton and Sumner, 1983). One such selection program resulted in development of an acid-tolerant alfalfa germplasm identified as AT and an acid-sensitive check germplasm identified as AS (Hartel and Bouton, 1989). Both germplasms are the result of divergent selection from a parental base of 10 released cultivars and/or germplasms, and are synthetics, each derived from 40 parents after an additional cycle of selection from the Cycle 2 acid selection and the Cycle 2 limed selection germplasms (Brooks et al., 1982). The same selection procedure (Brooks et al., 1982) was used to select the parents of AT and AS, except that seed of each Cycle 2 germplasm had been bulked across parentals before selection began in their respective soils (Hartel and Bouton, 1989).

The use of tissue culture to identify genotypes tolerant to Al is different from selection at the cell level to recover mutant cells resistant to Al, and subsequently regenerate resistant plants from these cells, as has been done with carrot, *Daucus carota* L.(Ojima and Ohira, 1982), and *Nicotiana plumbaginifolia* Viv. (Meredith et al., 1988; Conner and Meredith, 1985a,b). The former approach simply uses a callus assay to identify preexisting variability, whereas the latter depends on variability not present in the original explant source being expressed and selected in culture.

Both methods, however, depend on a correlation between response at the whole-plant level and at the cellular level. Although this approach has not been tested with alfalfa, previous work with other species suggests that whole-plant tolerance to Al and other metals is also expressed at the cellular level. Working with tomato (*Lycopersicon esculentum* L.), Meredith (1978) found whole-plant tolerance to Al is maintained at the callus level. This also appears to be the case with sorghum, *Sorghum bicolor* (L.) Moench. (Smith et al., 1983). Other work has shown that genotypic differences to Cu and Zn tolerance at the whole plant level were maintained in callus cultures of *Agrostis stolonifera* L. (Wu and Antonovics, 1978), and to Zn and Pb in cultures of *Anthoxanthum odoratum* (Qureshi et al., 1981). In addition, callus derived from acid-tolerant cultivars of alfalfa has also been shown to have a greater ability to grow on acidified medium than callus derived from non-acid-tolerant cultivars (Mezentsev et al., 1982).

The objective of this study was to test the in vitro performance of the AT and AS germplasms for tolerance to acid, Al-toxic soil conditions. If callus derived from acid, Al-toxic tolerant plants performs better on acidified, Al-toxic medium, then a callus assay could conceivably be used to identify parents for use in a breeding program.

MATERIALS AND METHODS

Media

Callus was grown on medium containing Blaydes salts and vitamins (Saunders and Bingham, 1975), supplemented with 2 mg L^{-1} each of indole-3-acetic acid (IAA), α-napthaleneacetic acid (NAA), and kinetin (KT). The pH was adjusted to 5.8, and the medium solidified with 3 g L^{-1} Gelrite (Kelly Lab., Tucker, GA). To simulate Al toxicity, the medium was modified according to the specifications of Conner and Meredith (1985c). The concentration of phosphate ions was reduced to 10 μM, and that of calcium to 100 μM. The pH was lowered to 4.0, unchelated iron was used, and the medium was solidified with 5 g L^{-1} of Gelrite. Four hundred μM of Al ions were added in the form of $AlCl_3$. The $AlCl_3$ was autoclaved separately, and added to the rest of the medium before pouring. Blaydes medium, modified as described but without any Al and solidified with 6 g L^{-1} Gelrite, was used as a control. These media are referred to as B+Al and B−Al, respectively (Table 1). All media were autoclaved at 121 °C for 20 min and dispensed into 100 by 20 mm disposable petri dishes in approximately 35 mL aliquots.

Plant Material

Seeds from the AT and AS populations were surface-sterilized with chlorine gas, and germinated on half-strength

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Table 1. Composition of Blaydes, B − Al, and B + Al media.

	Blaydes	B − Al	B + Al
		mg L^{-1}	
KNO_3	1 000.00	1 279.90	1 279.90
K_2HPO_4	20.40	–	–
NH_4NO_3	1 000.00	1 000.00	1 000.00
$MgSO_4 \cdot 7H_2O$	35.00	35.00	35.00
H_2BO_3	1.60	1.60	1.60
$MnSO_4 \cdot H_2O$	4.40	4.40	4.40
$ZnSO_4 \cdot 7H_2O$	1.50	1.50	1.50
KH_2PO_4	284.00	1.36	1.36
KI	0.80	0.80	0.80
$FeSO_4 \cdot 7H_2O$	23.60	23.60	23.60
Na_2EDTA	32.00	–	–
$Ca(NO_3)_3 \cdot 4H_2O$	347.00	23.60	23.60
KCl	65.00	33.10	33.10
NaCl	–	10.00	10.00
$AlCl_3 \cdot 6H_2O$	–	–	96.58
IAA	2.00	2.00	2.00
NAA	2.00	2.00	2.00
KT	2.00	2.00	2.00
Glycine	2.00	2.00	2.00
Thiamine·HCl	0.10	0.10	0.10
Nicotinic acid	0.50	0.50	0.50
Pyridoxine·HCl	0.10	0.10	0.10
Sucrose	30 000.00	30 000.00	30 000.00
Gelrite	3 000.00	5 000.00	6 000.00
pH	5.80	4.0	4.0

hormone-free MS medium (Murashige and Skoog, 1962). Seedling hypocotyls were placed on modified Blaydes medium to induce callus. All cultures were maintained at 25 °C, with a 16-h photoperiod. Illumination was provided by cool white fluorescent tubes (75–125 μmol photon m^{-2} s^{-1}). After 30 d, the callus from each seedling was split into six pieces as equally as possible. These were then weighed individually. Two pieces were transferred to separate plates of modified Blaydes medium, two to B−Al, and two to B+Al. The calli were transferred onto fresh media at 15-d intervals, with callus weight being recorded at each transfer, for a total of four transfers. Two experiments were run. In the first experiment, callus growth for 5 AT and 5 AS seedlings was evaluated on the three media as described. For the second experiment, callus was obtained from 30 seedlings from each germplasm, and the same procedure was followed.

Data Analysis

Callus weights after 2, 4, 6, and 8 wk were divided by the initial callus weight to obtain the fold weight increase over the respective period of time. Weight increases on B−Al and B+Al were then expressed as a percentage of the weight increase on Blaydes medium. Data were transformed with an arcsin transformation. Analysis of variance was performed on the transformed and untransformed data using the SAS Proc ANOVA (SAS, 1982) procedure. Media, alfalfa germplasms, and sampling dates were considered to be fixed effects, while replicates and genotypes within germplasm were considered to be random.

A subsequent analysis of variance was performed, as described above, for each of the four dates at which calli were weighed. Finally, an analysis of variance was conducted for each germplasm at each of the four weighing dates.

RESULTS AND DISCUSSION

Whereas Meredith et al. (1988) used sponges bathed in liquid media to support callus while selecting for Al tolerance, using Gelrite instead of agar allowed us to use solidified media (as agar gels poorly at low pH values). Callus was then easily recovered from the solidified media for weighing purposes.

The first experiment suggested that differences in Al tolerance between genotypes could be detected in cell culture. Because Blaydes medium was acidified and modified substantially (i.e., B−Al medium) to prevent the precipitation of the Al, we had to separate the effects of the medium modifications from the effects of the Al itself. Those modifications resulted in a depression of callus growth relative to that on Blaydes medium. The addition of Al resulted in an additional decrease in callus growth in the AS population, but not in the AT population, suggesting that breeding and selection of the AT germplasm has been effective in increasing Al tolerance. Callus from the AS and AT germplasms showed similar inhibition on B−Al medium (46.52 and 41.74% of control, respectively). However, there was a marked difference in performance when the calli were placed on B+Al medium. Callus growth from the AS germplasm was only 28.52% of control, while that of the AT germplasm was 41.76% of control. Thus, callus from the AT germplasm grew equally well on both B−Al and B+Al media. Callus growth of the AS germplasm was equivalent to that of AT on B−Al medium, but was depressed on the B+Al medium. These results were then tested in a second, larger experiment, which showed similar trends for Al tolerance (Table 2).

There was significant ($P < 0.01$) variability among genotypes within germplasms for Al tolerance, as evidenced by callus growth in the second experiment. The interaction of medium with genotypes within germplasm was also significant ($P < 0.01$), suggesting that the differential performance of the callus on media with and without Al is real, and can be used to identify genotypes with Al tolerance. A similar difference in the acid soil tolerance of the AS and AT germplasm was expressed in cell culture, but this difference did not become statistically significant until the 8th wk (Table 2). Prior to that time, callus growth of both germplasms was essentially equal on the B−Al medium.

Analyses of variance for the individual weighing dates showed highly significant $P < 0.001$) differences for genotype within germplasm at all dates, and a significant ($P < 0.01$) medium by genotype within germ-

Table 2. Average growth of callus from the acid-sensitive (AS) and acid-tolerant (AT) alfalfa germplasms on B − Al and B + Al media, as a percent of callus growth on Blaydes medium, during 8 wk.

	2 Wk		4 Wk		6 Wk		8 Wk	
	B − Al†	B + Al	B − Al	B + Al	B − Al	B + Al	B − Al	B + Al
AS	81.34 ± 6.31‡	75.74 ± 3.63	76.10 ± 7.19	67.41 ± 3.89	64.38 ± 6.31	55.03 ± 3.94	51.09 ± 5.23	45.47 ± 3.89
AT	84.48 ± 5.59	86.73 ± 5.28	73.06 ± 5.03	76.85 ± 5.20	62.85 ± 7.43	63.06 ± 5.69	62.91 ± 9.94	63.48 ± 8.01

† B − Al and B + Al are Blaydes medium without and with Al-toxicity. Media composition as in Table 1.
‡ Mean ± SE.

plasm interaction at the second weighing date. There was a significant ($P < 0.01$) effect due to germplasm by the 8th wk of culture. When data for each germplasm were analyzed at each date, there were significant ($P < 0.01$) effects due to genotype for both populations at all four dates. In addition, the genotype by medium interaction was significant ($P < 0.01$) for the AS germplasm at all dates, but was never significant for the AT germplasm. Effects due to replication were not significant ($P > 0.05$), except for the AT germplasm at the second weighing date, suggesting that any differences in the starting size of the callus did not affect the overall results.

It is clear that substantial variability still exists in both the AT and the AS germplasm. Although selection has been able to change the mean Al tolerance in the AT germplasm, plants sensitive to Al are still segregating, as evidenced by the fact that the most sensitive individuals in the AT germplasm are more sensitive than several individuals in the AS germplasm. This callus assay procedure has the added benefit that callus from individual plants can be easily replicated, whereas to replicate individual plants being tested for Al tolerance requires the time-consuming task of rooting cuttings. Also, with cell culture, Al tolerance was evaluated in a shorter period of time, and gave more controlled monitoring of the problem than is possible in a field experiment. Consequently it should be feasible to develop a strategy based on the use of callus culture to assist in identification of acid or Al-tolerant genotypes for use as parents in a breeding program. Toward this end, we are currently comparing the breeding progress obtained by using a callus assay to identify Al-tolerant parents, vs. using whole-plant screening techniques.

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