*INHERITANCE OF NITRATE REDUCTASE ACTIVITY IN ZEA MAYS L.**

By Robert L. Warner, R. H. Hageman, J. W. Dudley, and R. J. Lambert

AGRONOMY DEPARTMENT, UNIVERSITY OF ILLINOIS, URBANA

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Abstract.—Since the F_1 hybrid (B14 × Oh43) had been shown to have a higher (heterotic) level of nitrate reductase activity than either inbred parent (B14 or Oh43), studies were undertaken to determine the mode of inheritance. Standard methods for determining Mendelian inheritance were used to study segregation for level of nitrate reductase activity of individual plants. The genetic material used was the inbreds B14 and Oh43, F_1, F_1 backcrossed to both parents, F_2, F_3, and F_4 generations of the cross B14 × Oh43. The plant material was grown in the field and in growth chambers.

It was shown that the maize inbreds B14 and Oh43 differ at two loci that control the level of nitrate reductase activity. Each inbred is homozygous for a dominant or partially dominant allele at one locus and homozygous recessive at a second locus. The locus at which B14 carries a dominant allele carries the recessive allele in Oh43. Oh43 has both a higher *in vivo* rate of synthesis of nitrate reductase and higher *in vivo* and *in vitro* loss of enzyme activity (decay) than B14. Thus, the rates of both enzyme synthesis and decay are factors governing the level of nitrate reductase activity in corn. The data suggest that the heterotic level of nitrate reductase activity in the F_1 hybrid is the result of inheritance of qualities that gives it "intermediate" rates of enzyme synthesis and decay.

Introduction.—Single-locus control of enzymatic activity has recently been shown for each of several enzymes. However, the mechanism of control appears to be quite different for different enzymes. Feinstein *et al.*[1] and Courtright[2] proposed allelic regulator loci controlling the level of enzyme synthesis. Rechcigl and Heston[3] demonstrated that *in vivo* enzyme turnover rate was regulating the level of catalase activity and that some external factor regulated by a pair of alleles at a single locus was controlling the *in vivo* turnover rate. In addition many mutant enzymes can be distinguished from the wild type by loss of thermal stability *in vitro*. Presumably the reduced thermal stability may also indicate to some degree an increased *in vivo* turnover rate, hence a regulatory effect on enzymatic activity.

Although regulation of enzymatic activity by allelic differences in regulator loci, structural loci, and a locus controlling *in vivo* turnover rate has been demonstrated, no observation of multiple loci controlling enzymatic activity has been reported for higher plants. In view of the number of sites at which control of activity has been demonstrated in various organisms, the existence of multiple loci controlling enzymatic activity is to be expected.

This communication describes the inheritance of nitrate reductase activity in populations derived from two inbred lines of maize. These two inbreds, when

crossed, produced an F_1 hybrid that had a higher (heterotic) level of nitrate reductase activity than either parent.[4] Mechanisms are proposed by which separate loci may influence the observable nitrate reductase level in maize.

Materials and Methods.—The material included the parental inbreds B14 and Oh43, F_1, F_2, BCP_1 and BCP_2 generations, all of which were grown under field conditions at Urbana, Illinois, in 1965 and 1966. In addition, six selected F_3 progenies produced by selfing F_2 plants in 1965 were grown and assayed in 1966. A total of 43 random F_2 plants were selfed in 1966 and the F_3 progenies were evaluated for nitrate reductase activity as seedlings. The F_3 plants grown in 1966 were also selfed and the segregations based on nitrate reductase activity determined for the F_4 seedlings.

Field-grown maize plants were sampled individually at weekly intervals beginning 28 days after planting by removing the top, fully expanded leaf. Nitrate reductase extraction and assay procedures used have previously been described.[5] Where possible, the experiments were repeated with replicated separate samples.

Seedlings to be assayed were grown in a growth chamber at 29° and 3500 ft-c under a 15-hr photoperiod and at 21° during the 9-hr dark period; supplied with a pH 4.0 nutrient solution as follows (in mM): KH_2PO_4, 0.5; $MgSO_4$, 3.0; $Ca(NO_3)_2$, 5.0; KNO_3, 5.0; $(NH_4)_2SO_4$, 2.5; Fe (as Sequestrene 330, Geigy Agricultural Chemical Co., Yonkers, New York), 0.02; H_3BO_3, 0.023; $MnCl_2$, 0.046; $ZnSO_4$, 0.015; $CuSO_4$, 0.002; MoO_3, 0.002; and supported by Vermiculite (Zonolite Co., Chicago). For comparative purposes, seedlings were harvested 9 or 10 days after planting, which is the stage of maximal enzyme activity. Nitrate reductase was extracted from individual seedlings by a micromodification of the procedure used on field-grown plants.

Partially purified nitrate reductase was used for determination of pH and temperature optima and Michaelis (K_m) constants. The K_m values (NO_3^- and NADH) were obtained from conventional double-reciprocal plots.[6] Partial purification of nitrate reductase was accomplished with calcium phosphate gel absorption and ammonium sulfate precipitation (0–45%) as described by Evans and Nason.[7]

Results and Discussion.—Field studies in 1965 and 1966 indicated that inheritance of nitrate reductase activity was under relatively simple genetic control. Frequency distributions for the mean activity of five samplings of each plant in 1965 and three samplings of each plant in 1966 are presented in Table 1. In 1965, both inbreds and the F_1 had a small range of activity with no overlap of values of individual inbred plants with the F_1 or with each other, although in previous years Oh43 and B14 had similar activity levels.[8, 9] The two backcrosses had bimodal distributions, with each modal class approximately equal in size and roughly corresponding to the hybrid and the respective inbred in nitrate reductase activity. The activity distribution of the F_2 plants extended from the lowest observed with Oh43 to the highest found for the F_1. About half (26) of the F_2 plants were similar to the F_1 in activity level, while the remainder (24) had levels of activity similar to that observed for the inbreds. Six F_2 plants—two with low activity, one with intermediate low activity, and three with high activity—were selfed for study of F_3 segregations. Again in 1966, the frequency distributions for Oh43, B14, and the F_1 were over a small range and were very similar to those observed in 1965, except that all activities were higher. These higher levels are attributed to a more favorable environment for growth in 1966. The level of nitrate reductase activity in the two inbreds was nearly the same in 1966. This equality is consistent with the earlier evaluations of these two inbreds,[8, 9] but differs from the 1965 results when Oh43 gave lower activities. The backcross (BC) distributions in 1966 were again bimodal and the F_2 distri-

TABLE 1. *Distribution of the mean nitrate reductase activities of individual field-grown maize plants.*

	No. Plants in Each Generation					
					Backcross to:	
Range of activity* (1965)†	Oh43	B14	F_1	F_2	Oh43	B14
1.5						
3.0	19			4(*a*)		
4.5	1			4	13	
6.0		1		5	7	4
7.5		12		9(*b*)	3	16
9.0		7		2	7	3
10.5			5	5	13	9
12.0			14	17(*c*)	7	12
13.5			1	4(*c*)		5
15.0						
				F_3		
Range of activity* (1966)†	Oh43	B14	F_1	(*a*)	(*b*)	(*c*)
4.5				19	2	
6.0				23	5	
7.5				15	5	1
9.0	5				15	5
10.5	9	7			7	5
12.0	6	9			1	18
13.5		4				4
15.0			7			16
16.5			11			23
18.0			2			24
19.5						15
21.0						2

* In μmoles NO_2^-/gm fresh weight per hr (upper class limits).

† In 1965 Oh43, B14, F_1, F_2, and backcross plants were each sampled five times. Six F_2 plants were selected: (*a*) two lows, (*b*) one intermediate low, and (*c*) three highs; they were selfed to give three classes of F_3 plants. In 1966 Oh43, B14, F_1, and F_3 plants (from six selected 1965 F_2 plants) were each sampled three times.

bution was similar to that in 1965, except that certain individual F_2 plants were lower in activity than either inbred.

Distribution patterns of nitrate reductase activity observed with the F_2 and BC genotypes suggest that genetic control is either by a one-locus system with overdominance or by a two-locus system with dominance. With either model (one- or two-locus), bimodality in the backcross and $^1/_2$ or $^9/_{16}$ of the F_2 plants equal to or greater than the F_1 in activity is expected. Experimental techniques are available for distinguishing between these two models. The two-locus model predicts that when F_2 plants are selfed, the following genotypes and associated activity phenotypes should be produced: (1) double recessive homozygotes with activity lower than either B14 or Oh43; (2) single heterozygotes with activity comparable to that of the inbreds, whose selfed progeny would give a 3:1 segregation (inbred:double recessive); (3) double heterozygotes with activity as in the F_1, whose progeny would segregate as did the F_2; (4) single heterozygotes with activity equal to or higher than the F_1, whose progeny would give a 3:1 segregation (F_1:inbred); and (5) double dominant homozygotes with an activity level higher than the F_1.

Of the six F_2 plants selfed in 1965, the three high selections segregated approximately 3:1 for hybrid and inbred type; the intermediate low plant segre-

gated approximately 3:1 for an inbred and the double recessive class; while the two low selections appeared to be double recessive, as their activity level was lower than either inbred. These results strongly favored the two-locus model. In addition, F_4 seedling data confirmed the proposed genotypes of the F_3 plants and F_4 lines homozygous for high activity. Results of analysis of the progenies of the 43 F_2 plants selfed in 1966 showed 5 double dominant homozygotes, 13 segregating as in the F_2, 9 segregating 3:1 for high to inbred NRA, 3 Oh43 type, 9 B14 type, and 4 homozygous double recessives with an expected ratio of 1:4:4:3:3:1. The goodness of fit (χ^2) of 6.58 ($P = 0.2-0.3$) indicated an acceptable fit to the model. The basis for distinguishing between B14, Oh43, and double recessive homozygotes was the differential activity of the homozygous recessive seedlings and the differences in *in vitro* decay rates (Tables 4, 5, and 6) between B14 and Oh43.

Enzyme characterization studies were made with the five genotypes (Oh43, B14, F_1, homozygous double dominant, and double recessive) to determine whether the genes controlling nitrate reductase activity were regulating it by some physical difference in the enzyme or whether the regulation was due to the amount of nitrate reductase present in the plant. The first step was to partially purify nitrate reductase by calcium phosphate gel absorption followed by ammonium sulfate precipitation (0–45%) from each of the five genotypes. The results of these experiments are presented in Table 2. The degree of purification attained was approximately the same for all genotypes. Based on the similarities of reaction pH, temperature responses, and K_m values, there is no reason to believe that observed differences in activity of the five genotypes were due to physical differences in the enzyme, although no valid estimate can be made on turnover number, which is essential for a final judgment.

Freezing purified nitrate reductase preparations from Oh43 and double recessive genotypes resulted in a greater proportionate loss of activity (decay) than freezing preparations from the other genotypes, which suggests genotypic differences in reductase stability. Partially purified nitrate reductase from each of the five genotypes was incubated at 0° and 29° and assayed at intervals for 12 hours. The rates of enzyme decay for the five genotypes (calculated from the slope of the log nitrate reductase activity vs. time) are shown in Table 3.

TABLE 2. *Partial purification, activities, and characteristics of nitrate reductase from five maize genotypes.*

Genotype	Specific Activities[a]		Characteristics[c]			
			Reaction		K_m	
	Crude	Purified	pH[d]	Temp. (°C)[e]	NO_3^- ($\times 10^{-4}$ M)	NADH ($\times 10^{-5}$ M)
DD[b]	1.8	22.7	7.5	33	1.3	2.7
F_1	1.3	23.4	7.5	33	1.4	2.7
B14	0.7	13.8	7.5	33	1.3	2.7
Oh43	0.8	13.4	7.5	33	1.3	2.9
DR[b]	0.5	7.7	7.5	33	1.4	2.9

[a] Expressed as μmoles NO_2^-/mg protein per hr, and protein was determined according to Lowry *et al.*[10]

[b] Double dominant and double recessive, respectively.

[c] The characteristics were determined on the partially purified preparations and each value is the average of at least three preparations from each genotype.

[d] The pH range was varied from 6.0 to 9.0.

[e] The temperature range was varied from 24 to 46°.

TABLE 3. *In vitro inactivation (decay) rates* of partially purified nitrate reductase from nine-day-old seedlings.*

Genotype	Loss per Hour 29° (%)	Loss per Hour 0° (%)
DD†	15.4	2.3
F_1	23.1	4.0
B14	18.2	2.5
DR†	65.0	12.9
Oh43	97.0	12.2

* Computed from log nitrate reductase activity vs. time.
† Double dominant and double recessive, respectively.

The enzyme from homozygous double dominant, F_1, and B14 genotypes is relatively stable at 0°, decreasing only 2–4 per cent per hour, whereas activity from Oh43 and the double recessive decreased by 12–13 per cent per hour. At 29°, the enzyme from all five genotypes was much less stable than at 0°. However, the decay rates increased proportionately with Oh43 and the double recessive, which lost most of their activity within one hour, while activity from the double dominant, F_1, and B14 decayed 15–23 per cent per hour. There are differences in the *in vitro* decay rates of the enzymes prepared from the five different genotypes (Table 3). Undoubtedly the high *in vitro* decay rate is the cause of the very low activity in field-grown Oh43 compared with B14 in 1965, as there was a delay of several hours between homogenization and assay in 1965, while in 1966 this delay was greatly reduced.

The level of activity in the crude extracts varied with seedling age and genotype (Table 4). However, the patterns of activity were similar for the three genotypes except that the F_1 reached maximum activity earlier than the inbred parents. Stability (*in vitro*) varied with genotype, seedling age, and leaf position (Table 4). The effect of age is apparently due to physiological age, as the upper and lower leaves of the same plant gave different levels of activity and enzyme decay rates.

Although attempts were made to determine the causal factor(s) for the accelerated loss of enzyme activity *in vitro*, especially for the Oh43 preparations, no definitive answers were obtained. Since partial purification of the enzymes

TABLE 4. *The influence of seedling age and leaf position on the level of activity and in vitro stability at 0° of nitrate reductase from B14, Oh43, and F_1 genotypes.*

Seedling age (days)	Activity (μmoles NO_2^-/gm fresh weight per hr)* B14	Oh43	F_1	Stability† (% loss/hr) B14	Oh43	F_1
7	8.1	4.2	16.5	—	—	—
8	11.0	5.1	17.5	0.8	2.0	—
9	12.3	8.8	15.8	2.0	7.9	4.0
10	9.5	7.5	15.4	2.2	13.8	—
11	7.0	7.0	10.1	3.4	19.1	5.0
12	5.4	5.4	9.2	—	—	—
13	4.6	5.0	8.9	2.7	31.1	6.0
10‡	12.8	9.8	—	2.0	9.8	—
10§	10.3	4.6	—	4.4	26.0	—

* Total expanded leaf tissue of the seedlings was used in the age experiment.
† Computed from log of nitrate reductase activity vs. time.
‡ Upper leaf.
§ Lower leaf.

did not alter decay rate, soluble inhibitor(s) are not responsible for loss of activity. Addition of exogenous protein or the maintenance of protein concentration at higher levels than found in the crude extract during purification were of little benefit and tend to rule out proteases or critical mass of nitrate reductase as causal factors of decay. Mixtures of enzyme preparations, both crude and purified, gave inconclusive results with respect to accelerated or retarded rates of decay.

It appears that the enzyme preparation from Oh43 has some inherent property causing Oh43 reductase to be much less stable than that of B14. Whether this factor is a property of the Oh43 enzyme or some other factor that accumulates with leaf age, which is not removed by partial purification and has no effect on nitrate reductase from B14, cannot be clearly stated at this time. The most likely assumption is that the Oh43 enzyme has some property that makes it more susceptible to substances accumulating in aging leaves.

It has previously been shown that nitrate reductase activity fluctuates diurnally[11] and declines when plants are placed in the dark.[12,13] In an attempt to relate *in vivo* reductase decay rate to *in vitro* decay rate, seedlings were placed in a dark, 30° chamber. The plants were assayed for nitrate reductase activity at regular intervals for 12 hours and the enzyme decay rate was computed. Although the highest loss of activity was noted for the Oh43 seedlings *in vivo* (Table 5), there was less divergence among the genotypes in the rate of loss of enzyme activity *in vivo* than *in vitro*. The F_1 activity *in vivo* decay rate was only slightly less than the Oh43 activity decay rate, although the *in vitro* decay rate was similar to that of B14. No good explanation of this anomalous behavior of the F_1 with respect to *in vitro* and *in vivo* decay rates is available.

The differences in the *in vivo* decay rates may be an important factor in the relative nitrate reductase activity level observed in various genotypes, especially if one assumes that the enzyme is being constantly inactivated at the same rate in the light as in the dark. Therefore, experiments were conducted to determine if the *in vitro* decay rates can be used as a method of predicting *in vivo* differences of activity. Accordingly, an F_4 plant (with activity comparable to that of the inbreds) was crossed to B14 and Oh43 plants. Seedlings from the two new genotypes were assayed along with the parental and F_1 material for activity and the *in vitro* decay rates obtained for the enzymes from the parental lines (Table 6). Only the cross (Oh43 × F_4) gave heterotic levels of activity and the activity level was comparable to that of the F_1 hybrid. This shows that the F_4 plant was homozygous recessive and dominant at the respective loci and of the same activity genotype as B14 and that the *in vitro* stability permits the delineation of

TABLE 5. *Effect of seedling age on in vivo nitrate reductase activity decay rates* of Oh43, B14, and F_1 seedlings placed in the dark at 30°.*

Seedling age (days)	Per Cent Loss per Hour		
	Oh43	B14	F_1
8	10.9	3.8	8.3
9	10.8	2.6	9.2
10	11.6	5.1	9.8

* Computed from log nitrate reductase activity vs. time.

TABLE 6. *Nitrate reductase activity and in vitro 0° decay rates* of eight-day-old seedlings of F_4,‡ B14, Oh43, B14 × F_4, Oh43 × F_4 and B14 × Oh43 genotypes*

Material	Activity†	Per cent loss per hour
F_4	12.1	0.7
B14	13.1	0.7
Oh43	13.7	4.0
B14 × F_4	13.4	—
Oh43 × F_4	19.0	—
B14 × Oh43 (F_1)	19.1	—

* Computed from log nitrate reductase activity vs. time.
† μmoles NO_2^-/gm fresh weight per hr.
‡ This plant exhibited a level of activity comparable to that of the inbreds and could have a homozygous recessive and dominant genetic composition, with respect to nitrate reductase activity, characteristic of either parent. The *in vitro* stability predicts that F_4 was the B14 genotype; this was verified by the heterotic level of nitrate reductase activity observed in the Oh43 × F_4 cross.

its genotype. Consequently, the cross to B14 would not be expected to result in a heterotic activity level. These data in conjunction with those presented in Table 3 further support the concept that *in vitro* stability of reductase is in some way related to the activity level in the plant.

Since nitrate reductase activity fluctuates diurnally and the activities of B14 and Oh43 decay at different rates in the dark, the rate at which reductase is produced in the light may provide the counterbalance to maintain the activity level of Oh43 and B14 at approximately the same level. Experiments (details given in legend of Fig. 1) established that Oh43 seedlings exhibited the greatest initial rate of increase of activity and the rate of increase was nearly linear for four hours. It is presumed that this initial period represents the genetic potential for synthesis of reductase. The subsequent decline is attributed to a new balance between rate of synthesis and decay. Whether this was due to an acceleration of decay or a decrease in rate of synthesis is not known. Nitrate level in the tissue at the end of the experiment was adequate for induction. While the B14 and F_1 material initially had lower rates of increase of activity than Oh43, they

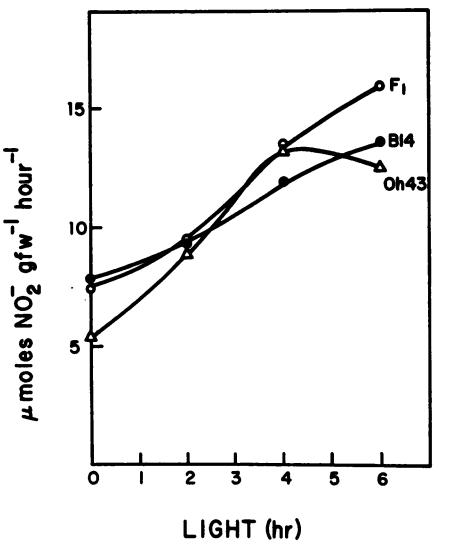

FIG. 1.—Rate of increase (synthesis) of nitrate reductase in 8-day-old seedlings of the three genotypes, Oh43, B14, and F_1. The seedlings were placed in the dark at 30° for 14 hr prior to their exposure to light.

tended to maintain this rate over the six-hour period. The rate of increase in the F_1 was always greater than for the B14. The ranking of the three genotypes at 0 time (after 14 hr of dark treatment) is consistent with the *in vivo* decay rates presented in Table 5. These results support the contention that the rates of both enzyme synthesis and decay are factors in governing the nitrate reductase activity level in plants.

These data suggest that the heterotic level of activity in the F_1 hybrid is the result of inheritance of qualities that gives intermediate rates of enzyme synthesis and decay. Although the F_1 more nearly resembles B14 in rates of enzyme synthesis and resembles Oh43 in *in vivo* rates of decay, the interaction of rates of synthesis and decay with time would permit the heterotic level of activity in the F_1.

Since nitrate reductase is a substrate-inducible enzyme, it is presumably under regulation by inducers and repressors; thus the rate of synthesis of this enzyme may be related to a regulator gene. Pateman and Cove[14] have found two loci involved in nitrate reductase control in *Aspergillus nidulans*, one locus being a regulator site and the other a structural site. They found regulator mutants of two types; one type prevented induction and the other caused constitutive nitrate reductase synthesis. The structural gene mutants lacked normal activity. Their findings would appear to agree with the observations with higher plants presented here both in terms of two loci being involved and also in the possible mechanism or function of these loci.

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