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Nitrate reductase activity, biomass, yield, and quality in cotton in response to nitrogen fertilization

Actividad de la nitrato reductasa, biomasa, rendimiento y calidad en algodón en respuesta a la fertilización nitrogenada

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Abstract. In the production of cotton (*Gossypium hirsutum* L.), nitrogen fertilization is one of the most costly crop practices, but important to reach high yields. However, high nitrogen (N) content in plants does not always translate into a high fibre production. One way of assessing the efficiency of the N fertilizer is through the enzymatic activity of the nitrate reductase (NR). This is a key enzyme in N assimilation, whose activity is regulated by a number of endogenous and exogenous factors that determine yield. The aim of this study was to assess the effect of N fertilization on yield, fibre quality, biomass, and NR enzymatic activity in vivo in the cotton variety Fiber Max 989. The evaluated application rates were 0, 50, 100, and 150 kg/ha of N, using urea as a source (46% N) in a randomized-block design with three replicates. At harvest, the maximum yield of seed cotton and the greatest accumulation of total foliar biomass through time was reached after applying 150 kg N/ha. The different N-application rates did not affect the components of cotton-fibre quality. The activity of endogenous NR was greater on plants where 150 kg N/ha were applied. The highest cotton yield and N contents were obtained on these plants. Therefore, the NR activity *in vivo* could be used as a bioindicator of the N nutritional level in cotton.

Keywords: *Gossypium hirsutum* L.; Nitrogen assimilation; Yield.

Resumen. En la producción de algodón (*Gossypium hirsutum* L.), la fertilización nitrogenada es importante para alcanzar altos rendimientos. Sin embargo, un alto contenido de nitrógeno en la planta no siempre se traduce en una alta producción de fibra, lo que hace que esta práctica sea una de las más costosas para el cultivo. Una forma de valuar la eficiencia de la fertilización nitrogenada es a través de la actividad enzimática de la nitrato reductasa (NR) que es la enzima clave en la asimilación del Nitrógeno (N). Esta enzima es regulada por una serie de factores endógenos y exógenos que son determinantes en la producción. El objetivo del presente estudio fue determinar el efecto de las dosis de N en la producción, calidad de fibra, biomasa y actividad enzimática de la NR "in vivo" en la variedad de algodón Fiber Max 989. Las dosis evaluadas fueron 0, 50, 100 y 150 kg/ha de N (utilizando como fuente Urea: 46% de N), las cuales se distribuyeron en un diseño de bloques completamente al azar con tres repeticiones. Al realizar la cosecha, la máxima producción de algodón y la mayor acumulación de biomasa total foliar a través del tiempo se obtuvieron al aplicar 150 kg N/ha. Las diferentes dosis de N aplicadas no afectaron los componentes de calidad de la fibra de algodón. La actividad de la NR endógena fue mayor en las plantas donde se aplicaron 150 kg N/ha, las que de todos modos obtuvieron el mayor rendimiento de algodón y contenido de N. La actividad enzimática NR "*in vivo*" se podría utilizar como un bioindicador del estado nutricional del nitrógeno en algodón.

Palabras clave: *Gossypium hirsutum* L.; Asimilación de nitrógeno; Rendimiento.

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INTRODUCTION

Fertilization is one of the most costly practices of cotton cultivation. They account for around 50% of the production expenses. This is an efficient way of supplying essential nutrients to a growing crop (Estrada et al., 2008; Shivamurthy & Biradar, 2014). Nitrogen (N) is essential in growth and development of plants because it is one of the chief constituents of amino acids and proteins. Crops require more N than other nutrients; this element is considered the main nutrient limiting agricultural production of arid regions in recent years (Paungfoo-Lonhienne et al., 2008; Hou et al., 2009; Mind et al., 2014).

As with most cultivated species, growth and yield of cotton cultivars depend on N availability during the crop cycle. Controlled N applications can increase crop yield and soil quality (Palomo et al., 2002; Erhart et al., 2005; Haq et al., 2014). Higher application rates of N fertilization increase the strength, length and percentage of fibre, and the seed index in cotton (*Gossypium hirsutum* L.) (Palomo et al., 2003; Mind et al., 2014).

It is important to monitor the crop N status so that management changes can be made to optimize yield and quality of cotton crops. It is considered than N applications in the range from 67 to 112 kg/ha are sufficient to obtain maximum yields in cotton crops. Very high N application rates can lower crop yield because of the excessive vegetative growth and duration of the crop cycle. Current studies are focused in highlighting the optimal N rate for cottons crops, because this condition can vary due to different climates and soil types in the production zones (Jost & Cothren, 2000; Li et al., 2014).

Recent studies have been made on plant metabolic changes caused by stress (e.g., nutrient deficiency, toxicity). These metabolic pathways are studied using biochemical markers such as enzymes (Romero, 1995). Plant N metabolism involves the (1) uptake of nitrate (NO_3^-), (2) reduction of nitrates (NO_2^-), (3) conversion of NO_2^- to ammonium (NH_4^+), and (4) incorporation of NH_4^+ into organic compounds (Stitt, 1999). Nitrate reductase (NR) is an oxidoreductase enzyme involved in plant N assimilation. It is the first enzyme that is involved in N-NO_3^- assimilation. Therefore, NR is considered the key enzyme in the overall N-assimilation process, and it is essential for plant life (Sagi et al., 1998; De la Haba et al., 2001; Kouadio et al., 2007).

Nitrate reductase is the rate limiting enzyme in N assimilation, and plays a key point on metabolic regulation in crops. This enzyme is associated with plant growth and development of crops, such as cotton. Plants exposed to stress decrease their protein synthesis, and produce a fall in the nitrate reduction activity caused by the low nitrate flux. It has been demonstrated that seed cotton yield and yield components are severely affected by stress (De la Haba et al., 2001; Costa et al, 2008; Ananthi & Vijayaraghavan, 2012).

The aim of this study was to evaluate crop yield, cotton fibre quality and activity of the enzyme NR *in vivo* in commercial cotton, exposed to four different N treatments at different stages of plant development.

MATERIALS AND METHODS

Crop management and experimental design. The cotton variety Fiber Max 989 was sown in April 2011 in Torreón, Coahuila, México, under open-field conditions. The effect of four N application rates (0, 50, 100, and 150 kg/ha) were studied using urea (46% N) as the N source. Seeds were sown in rows 75 cm apart and plants were spaced 12 cm in the same row. There was a population of 110000 plants/ha. We used a completely randomized block design with three replicates. Soils were analysed at the study area: pH (8.06 without salinity or sodium), nitrate (14.5 ppm); phosphorus (18.9 ppm); potassium (193 ppm); manganese (3.15 ppm); Zinc (0.91 ppm), copper (0.7 ppm), and iron (2.20 ppm); total carbonates (14.5%), and organic matter (1.56%).

Plant sampling. Sampling was made on the fifth leaf of the main shoot at 74, 94, and 136 days after sowing (DAS) during early flowering, full flowering, and boll opening, respectively. Collected samples were washed once with running water, once with distilled water, and finally with deionised water. One portion of the sampled plant material (0.15 g of leaf) was used fresh for the analysis of the NR enzyme. The remaining material was dried at room temperature in the shade and afterwards in an oven at 60 °C during 24 h. Thereafter, samples were ground in a Wiley mill with a stainless-steel chamber using a 20-mesh screen size for N determinations.

Determination of foliar biomass, yield, and fibre quality. Leaf biomass production was determined at 77, 99, and 146 DAS, the latter reflecting the total leaf biomass in g/m^2. To establish yield values, 6 m of each replicate were harvested and weighed. For yield components and fibre quality, random samples were taken from 20 bolls per replicate to determine boll weight, seed index, and fibre percentage. Cotton fibre was separated from the seed, and the length, strength, fineness, and uniformity of the fibre was quantified.

Plant analysis

Assay and determination of the in vivo enzymatic activity of the nitrate reductase. The *in vivo* NR activity (EC 1.6.6.1) was determined following Jaworski (1971). Leaf blades were cut into 5-mm sections (100 mg) and placed into 10 cm^3 of incubation buffer [(100 mM K-phosphate buffer, pH 7.5, and 1% (v/v) propanol)]. The samples were infiltrated and the intracellular spaces of the tissues were flushed with buffer using a vacuum (0.08 MPa). After 5 min, the vacuum was released and the samples were re-evacuated, incubated at 30 °C in

darkness for 1 h, and finally placed in a boiling water bath to stop the NR activity. The resulting nitrite concentration was determined spectrophotometrically at 540 nm in a reaction mixture containing 2 cm^3 of extract, 2 cm^3 of 1% (m/v) sulfanylamide in 1.5 M HCl, and 2 cm^3 og 0.02% (m/v) N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.2 M HCl. ($NR+NO_3^-$) was determined following the same method, but using a modified incubation buffer containing 50 mM KNO_3. The endogenous nitrate reductase activity (NRA) and the NRA induced by NO_3^-, or/and Mo were also determined using a modification of the incubation buffer containing 20 mM $NaMoO_4$ or 50 mM KNO_3 plus 20 mM $NaMoO_4$. The resulting nitrate concentration was also determined spectrophotometrically.

Determination of nitrogen. A subsample of 0.1 g dry weight was digested with sulphuric acid and H_2O_2 following Wolf (1982). After dilution with deionised water, a 1 mL aliquot of the digest was added to the reaction medium [(5% potassium sodium tartrate, 100 μM sodium phosphate and 5.4% (w/v) sodium hydroxide)] containing 15/0.03% (w/v) sodium alicylate/sodium nitroprusside and 5.35% (v/v) sodium hypochlorite. Samples were incubated at 37 °C for 45 min and organic N was measured by spectrophotometry at A_{630} as performed by Baethgen & Alley (1989). The results were expressed as a percentage.

Statistical analysis. All data were subjected to an analysis of variance. Differences between treatment means were compared using the LSD at the 0.05 probability level. Levels of significance were represented by * at $p<0.05$, ** at $p<0.01$, *** at $p<0.001$ and NS: not significant.

RESULTS AND DISCUSSION

Dynamics of the accumulation of leaf biomass. Nitrogen is the most widely used nutrient as a fertilizer, in high demand for cultivated plants (Weinhold et al., 1995). The accumulation of leaf biomass presented a progressive accumulation dynamic through the cultivation stages, according to the different rates of N applied, (Fig. 1). Plants fertilized at a rate of 150 kg N/ha reached the greatest total foliar biomass, which coincided with the highest cotton yield. Low N availability can translate into low biomass accumulation, findings that agree with those of Chen et al. (2008), which in turn can result in poorer yields. The lowest total leaf biomass in the present experiment was recorded in control plants, reflecting that an inadequate N supply often leads to a lower leaf area, photosynthesis and biomass production (Fernández, 1996), ultimately reducing yield and fibre quality (Reddy et al., 2004). Greater total biomass accumulation was found in the treatment of 50 kg N/ha, as opposed to the control; cotton yield was reduced at this N fertilization (Fig. 1), indicating that although growth responded well to N supply (Gerik et al., 1998), fibre production did not always increase because of raising the N-application rate (Boquet et al., 1994).

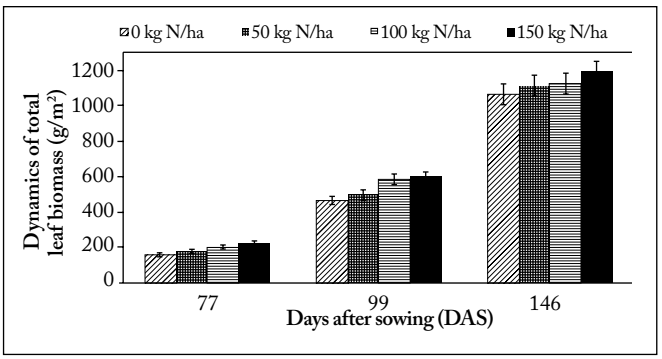

Fig. 1. Influence of different N-application rates on biomass accumulation in cotton.
Fig. 1. Influencia de las diferentes dosis de aplicación de N sobre la acumulación de biomasa en algodón.

Yield. In cotton productivity, quality, and profitability, N fertilization is a determining factor (Gerik et al., 1998). In our study, the different N dosages had a significant effect on seed and lint cotton (Table 1). The best yield resulted in the highest N-application rate (150 kg/ha) with an 11% increase over the control. Meredith et al. (1997) found that new cultivars respond better to higher rates of N as opposed to old varieties. These results disagree with those of Bell et al. (2003) who mentioned that high N rates can reduce yield; in their study, the rate of 100 kg/ha boosted yield 0.7% higher than the control. Nitrogen is essential for photosynthetic activity (Reddy et al., 1996), stimulates mobilization and accumulation of metabolites in developing bolls, controls growth, and impedes shedding of flower buds and developing bolls, thereby increasing their number and weight (Sawan et al., 2001). Plants given a rate of 50 kg N/ha registered 12.6% less yield than the control. This trend might be attributable to the influence of a number of factors that affect crop development, such as: soil (1) compaction, which restricts root penetration; (2) water supply; (3) air; and (4) nutrient availability (Chagas et al., 1994). The different application rates of N showed significant differences only in boll weight, where the highest values resulted from the application of 150 kg N/ha, while the lowest ones were found in the control. The percentage of fibre did not differ significantly among treatments. However, as the lowest value was recorded at the rate of 150 kg N/ha and the control registered the highest value, this might indicate that N enlarged the boll size due to an increase in the individual weight of the seeds, with a consequent reduction in the percentage of fibre. The seed index did not significantly fluctuate with the different N-application rates (Table 1). These results agree with those of Palomo et al. (2002).

Table 1. Cotton yield and its components at different N-application rates.
Tabla 1. Rendimiento del algodón y sus componentes por efecto de la aplicación de diferentes dosis de N.

N rate	Cotton yield (kg/ha)		Fibre	Boll weight	Seed
(kg/ha)	Seed	Lint	(%)	(g)	index (g)
0	4777 ab	2166 ab	45.3 a	5.3 b	8.7 a
50	4234 b	1891 b	44.7 a	5.4 ab	8.7 a
100	4809 ab	2169 ab	45.1 a	5.6 ab	8.6 a
150	5290 a	2268 a	42.9 a	5.7 a	8.5 a

Means with the same letters within the same column are not statistically different (DMS, 0.05).

Table 2. Cotton-fibre quality at different N-application rates.
Tabla 2. Calidad de la fibra de algodón por efecto de diferentes dosis de aplicación de N.

N rate (kg/ha)	Length		Strength	Fineness	Uniformity
	inches	(mm)	(g/tx)	(micronair)	(%)
0	1 3/32 a	27.9 a	27 a	4.6 a	82.5 a
50	1 3/32 a	27.7 a	26.7 a	4.5 a	82.9 a
100	1 1/8 a	28.2 a	26.9 a	4.5 a	83.2 a
150	1 3/32 a	27.2 a	26.9 a	4.5 a	82.1 a

Means with the same letters within the same column are not statistically different (DMS, 0.05).

Fibre Quality. The N-application rates did not significantly affect the cotton-fibre quality (Table 2). These results disagree with those of Palomo et al. (2003), where the quantity of applied N affected fibre length, strength, and fineness. However, the highest strength and fineness values were in the control, and the lowest values corresponded to 50 kg N/ha. In fineness, the rates of 50, 100, and 150 presented equal values. The greatest length and uniformity of the fibre resulted from a rate of 100 kg N/ha in contrast to the rate of 150 kg N/ha, which gave the minimum values. Some studies have demonstrated that N significantly affects fibre length and strength (Bauer et al., 1993). According to Estrada et al. (2008), fibre quality depends on the prevailing conditions during the growth period of the boll. On the other hand, the minimum requirements of the textile industry for fibre quality are: minimum length of 26.7 mm, fineness of 3.5 to 4.9 micronair, and strength between one degree average and very resistant 26 to 31 g/tex. All quality components were within the parameters demanded by the textile industry.

Enzymatic activity of nitrate reductase *in vivo*: early flowering, full flowering, and boll opening. The limiting step for N assimilation (reduction of NO_3^- to NO_2^-) is regulated by the nitrate reductase (Sivasankar & Oaks, 1996). In general, the endogenous NR activity on the three sampling dates was higher in cotton plants that received 100 kg N/ha, while the lowest values resulted in the control. In comparison with the cotton yield, as the endogenous enzyme activity declined, yield also declined. Plants receiving 50 kg N/ha had an enzymatic activity similar to that of plants applied with 150 kg N/ha; however, this similarity was reflected neither in the yield of cotton nor in the accumulation of total leaf biomass. On inducing the enzyme NR with NO_3^-, the plants showed a clear need for this substrate, indicating a higher NR activity in the three crop samplings for all the application rates. These results coincide with those of Redinbaugh & Campbell (1991). Crawford (1995) found that NR activity changed when altering the NO_3^- concentration in the growth medium. It bears mentioning that the greater endogenous NR activity induced with NO_3^- declined. After the samples were infiltrated with molybdenum (Mo), the NO_2^- concentration increased, reflecting greater enzymatic activity when induced by Mo. This activity was even greater than that stimulated with nitrates, underlining the importance of the Mo cofactor for activating NR and for converting nitrates to nitrites (Zimmer & Mendel, 1999). These results coincide with those of Lavon & Goldschimdt (1999), where the infiltration with Mo directly affected NR activity. The values of NR activity induced with nitrates and infiltrated with Mo at the same time showed the general need of plants for both nitrates and Mo to activate the enzyme, and thus make use of cellular N. It should be emphasized that, at early flowering, endogenous NR activity

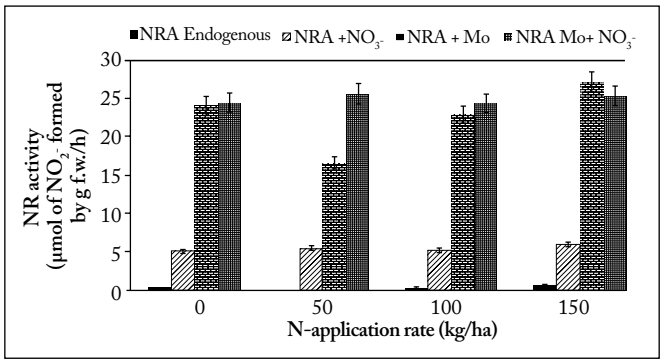

Fig. 2. Effect of the N-application rate on the nitrate reductase activity (NRA) *in vivo* at early flowering in cotton (74 DAS).
Fig. 2. Efecto de la aplicación de diferentes dosis de N sobre la actividad de la nitrato reductasa *in vivo* a principios de floración en algodón (74 DDS).

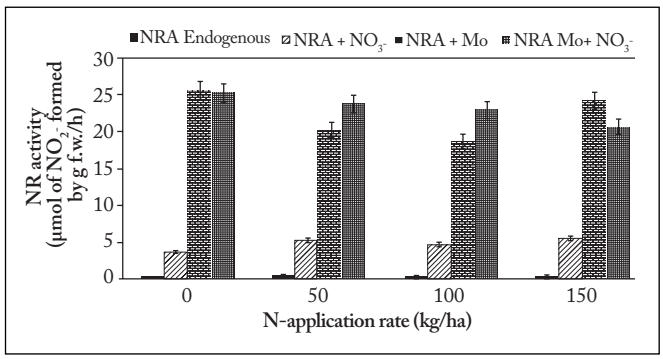

Fig. 3. Effect of the N-application rate on the nitrate reductase activity (NRA) *in vivo* at full flowering in cotton (94 DAS).
Fig. 3. Efecto de la aplicación de diferentes dosis de N sobre la actividad de la nitrato reductasa *in vivo* en completa floración en algodón (94 DDS).

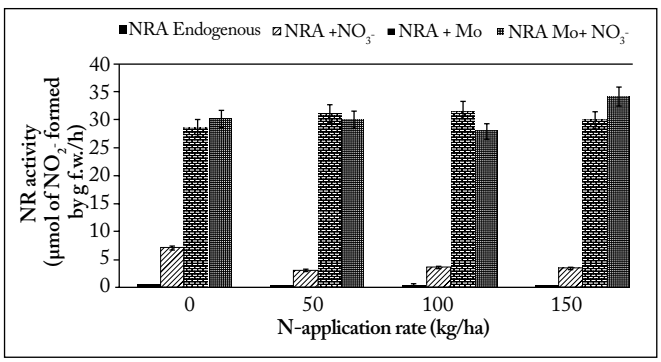

Fig. 4. Effect of the N-application rate on the nitrate reductase activity (NRA) *in vivo* at boll opening (136 DAS).
Fig. 4. Efecto de la aplicación de diferentes dosis de N sobre la actividad nitrato reductasa *in vivo* en apertura de capullos en algodón (136 DDS).

in the control reached the lowest value whereas 150 kg N/ha gave the highest value (Fig. 2). At full flowering of the crop, endogenous enzymatic activity increased (control, at the rates of 50 and 100 kg N/ha) with respect to early flowering, when the rate of 150 kg N/ha resulted in a slight decline in activity (Fig. 3). Nevertheless, full flowering of cotton coincides with the greater endogenous NR activity. Carvalho et al. (2006) noted that the presence of flowers and fruits in some plants accelerate and increase the endogenous NR activity; this is coincident with the stage of the greatest presence of flowers and the formation of cotton bolls. At the boll-opening stage, NR activity presented higher induction of nitrates, Mo, and the combination of both factors than at early and full flowering, while endogenous NR activity diminished (Fig. 4). This was presumably because the crop was in the maturation phase, and furthermore the NR activity varied according to the physiological age of the tissue, water status, light intensity, N source, temperature, Mo level (Mondy & Munshi, 1993), iron (Sikora & Cieslik, 1999), and sodium (Tarakcioglu & Inal, 2002).

N concentration. The greater N concentration at early and full flowering resulted from the N-application rates of 100 and 150 kg/ha. The lower value of leaf N at early flowering was found after applying 50 kg N/ha. At full flowering, the minimum value was registered on the control. Increased N fertilization raised the N levels in the plant, as it was demonstrated in other studies (Chen et al., 2008). In the boll-opening stage, the highest N concentration was recorded in the control and after applying 150 kg/ha, whereas the lowest value was found with 100 kg N/ha (Fig. 5). In general, the N concentration declined with advancement of the growing cycle. The greatest demand was at early flowering; afterwards it diminished at full flowering, finally reaching the lowest levels at the boll-opening stage (Fig. 5). The N concentration followed an individual trend for each applied rate, and was correspondingly associated with yield. The rate of 150 kg/ha gave the highest yield and the highest N

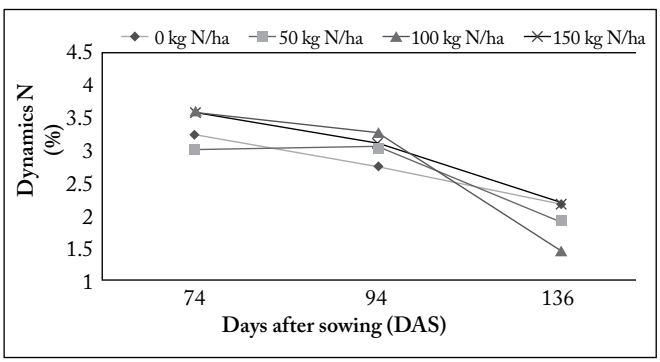

Fig. 5. Influence of the N-application rate on leaf N concentration in cotton (early flowering, full flowering, and boll opening).
Fig. 5. Influencia de la aplicación de diferentes dosis de N en la concentración de N foliar en algodón (inicio de floración, completa floración y apertura de capullos).

concentration except for the rate of 100 kg, which was slightly higher at the full-flowering phase (Fig. 5). Jones et al. (1991) reported that the sufficiency rates for early flowering in cotton ranged from 3.5 to 4.5% of N, and for the stage of full flowering from 3.0 to 4.30% of N. In the present study, the values of the rates of 100 and 150 kg N/ha were within the sufficiency ranges reported by Jones et al. (1991). At early flowering, however, 50 kg N/ha and the control presented lower N concentrations than the range reported by the previous authors. Rates of 50, 100, and 150 kg/ha gave N concentration values within the range reported by Jones et al. (1991) for the full-flowering stage (Fig. 5). The control, however, fell below that range (Fig. 5)

CONCLUSIONS

The optimum N-application rate was 150 kg/ha, as it allowed reaching maximum yield in seed cotton and lint, as well as the greatest accumulation of total leaf biomass over time. The different N rates applied did not affect the fibre-quality components of cotton. The enzymatic activity of endogenous NR was greater on plants where 150 kg N/ha was applied than when the greatest cotton yield and N content were reached. On the other hand, during the three study stages, the different N-application rates reflected the physiological needs for either nitrates or Mo or the combination of the two (i.e., NO_3^- + Mo) to activate the enzyme, and express its greatest activity in N metabolism. Therefore, the enzymatic activity of the nitrate reductase *in vivo* could be used as a bioindicator of the nutritional state of N in cotton.

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