Inhibition of gibberellin biosynthesis by nitrate in *Gibberella fujikuroi*

Rocío Sánchez-Fernández*, Javier Avalos, Enrique Cerdá-Olmedo

Departamento de Genética, Universidad de Sevilla, Sevilla, Spain

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Abstract Gibberellin production in *Gibberella fujikuroi* starts upon exhaustion of the nitrogen source. To determine the role of nitrate and ammonium in the regulation of gibberellin biosynthesis we have isolated mutants that cannot use nitrate as a nitrogen source. Nitrate inhibited partially the production of gibberellins in mutants devoid of nitrate reductase activity. The inhibition occurred whether nitrate was added before or after the onset of gibberellin production. Addition of tungstate to the wild type mimicked the results with nitrate reductase mutants. We conclude that nitrate inhibits gibberellin biosynthesis by itself, independently of the intracellular signal that conveys nitrogen availability.

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Key words: *Gibberella*; Gibberellin; Nitrate; Tungstate; Inhibition

1. Introduction

The gibberellins, plant hormones with practical applications in agriculture and brewing, are present in small concentrations in plant tissues and in large amounts in the culture media of *Gibberella fujikuroi* [1–4]. This fungus is the industrial source of gibberellins and a useful subject for research on the gibberellin biosynthetic pathway [5,6].

Gibberellin biosynthesis is strongly influenced by the growth conditions of *Gibberella*. A high carbon to nitrogen ratio is required for gibberellin production in batch cultures [7]. In our standard strain, the onset of gibberellin production is not a general consequence of unbalanced growth, but starts only upon exhaustion of the nitrogen source [8]. Nitrate, ammonium, and L-glutamine block gibberellin biosynthesis, independently of whether present from the beginning or added to a producing culture [8]. These three nitrogen sources may act through a common signal, since nitrate and glutamine produce ammonium in the cell. Use of L-methionine-DL-sulfoximine, an inhibitor of glutamine synthetase, suggests that the intracellular signal is glutamine or a compound derived from it [9]. All nitrogen sources tested, including urea and many amino acids, are effective inhibitors [10]. A partial nitrogen repression was found in deregulated strains that produce gibberellins in the presence of a nitrogen source [11,12].We have investigated the relation between nitrate assimilation and the regulation of gibberellin biosynthesis.

*Corresponding author. Present address: Laboratorium voor Genetika, Rijksuniversiteit Gent, B-9000 Gent, Belgium. Fax: (32) (9) 2645349. E-mail: rosan@genwet1.rug.ac.be

2. Materials and methods

Strain IMI58289 is a gibberellin-producing wild type of *Gibberella fujikuroi* that belongs to mating group C, a separate genetic species, and was received from the Commonwealth Mycological Institute, Kew, England. This strain and mutants derived from it were grown in 500-ml Erlenmeyer flasks with 250 ml minimal broth [8,13] at 30°C in the dark in an orbital shaker (150 rpm). The standard nitrogen source was NH_4NO_3 (6 mM, 0.48 g l^{-1}). Replacement by $NaNO_3$ at 1 g l^{-1} or NH_4Cl at 0.64 g l^{-1} maintained the total nitrogen concentration. When indicated, the medium contained other nitrogen source concentrations or tungstate, prepared as a filter-sterilized 0.5 M solution of Na_2WO_4 in distilled water.

Gibberellin concentrations in the media were estimated fluorometrically [14] using gibberellic acid (Sigma, St. Louis, MO, USA) as a reference. Mycelial mass was determined by weighing mycelial samples dried for 24 h at 105°C. Nitrate in the medium was determined with nitrate reductase (kit No. 905568, Boehringer Mannheim, Mannheim, Germany). For measurements of nitrate reductase activity, the mycelia were filtered, washed, frozen at −80°C, and extracted as described by [15]; the clear supernatant was kept in ice until the determination of enzyme activity [16].

Mutants unable to utilize nitrate or nitrite as nitrogen source were isolated after exposing spores to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.2 mg ml^{-1}) for 1 h, allowing them to grow on DG agar [17] with L-asparagine (18 mM) instead of $NaNO_3$, and testing the resulting colonies for growth on DG agar with asparagine, $NaNO_3$ (36 mM), or $NaNO_2$ (10 mM) as the only nitrogen sources. Colonies that grew with asparagine, but not with nitrate or nitrite after four subcultures, were checked for growth with NH_4Cl (36 mM) or hypoxanthin (9 mM). The nitrogen source concentrations correspond to 0.5 g l^{-1} of N atoms, except for NO_2Na, which was used at 0.14 g l^{-1} of N atoms because of its toxicity.

3. Results

3.1. Mutants defective in nitrate assimilation

Wild-type mycelia grown with ammonium as nitrogen source contained a considerable nitrate reductase activity (Fig. 1); at the time of the assay, the nitrogen source was already exhausted [8]. Addition of nitrate induced an even higher activity; this increase did not occur when nitrate was accompanied by ammonium.

Mutants unable to utilize nitrate as a nitrogen source were isolated by testing the ability of 9700 colonies produced by mutagen-exposed wild-type spores to grow on media with nitrate or asparagine. The mutants (Table 1) were classified according to their ability to utilize various nitrogen sources [18].

Three mutants were specifically defective for nitrate assimilation. Their in vitro nitrate reductase activity was nearly undetectable in all conditions tested. They grew normally with ammonium as the nitrogen source, but practically nothing with nitrate (Fig. 1). The same slight growth was found in the absence of a nitrogen source in the medium and may be attributed to the nitrogen reserves in the inoculum or to contaminating traces of ammonium in the medium. Growth with

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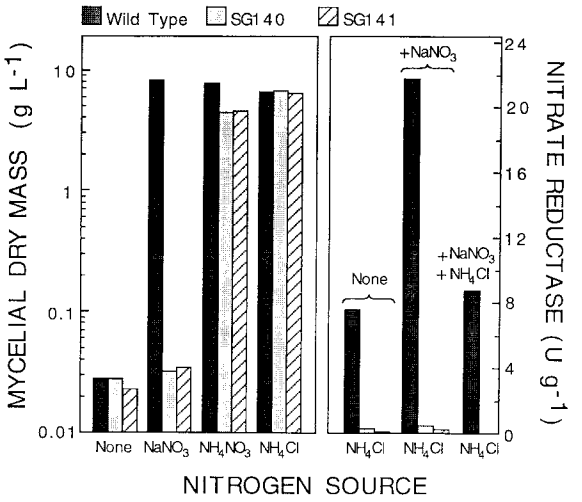

Fig. 1. Growth and nitrate reductase activity of the wild type and two nitrate reduction mutants. Left: Mycelial dry mass after 15 days growth in minimal broth with no nitrogen source or with various nitrogen sources used at equal concentrations of total nitrogen. Right: Nitrate reductase activity after growth for 75 h in minimal broth with NH_4Cl (12 mM); at the age of 72 h some of the cultures received additions of $NaNO_3$ (50 mM) and NH_4Cl (50 mM).

NH_4NO_3 confirmed that the mutants were able to use the ammonium, but not the nitrate.

Two mutants were defective in nitrite assimilation; they grew much better with NH_4Cl than with NH_4NO_3, presumably because they were inhibited by the nitrite made from nitrate. A mutant that could not utilise hypoxanthin must be affected in the biosynthesis of the molybdenum cofactor present in nitrate reductase and other redox enzymes. One mutant, SG149, exhibited an unexpected hypersensitivity to nitrite. Two asparagine-requiring strains were isolated in the same screening.

Two mutants, SG151 and SG152, unable to use nitrite as nitrogen source, were isolated after mutagenesis of mutant strain SG140; their assimilation of nitrate was doubly blocked: to the original lack of nitrate reductase was added another defect in nitrite reductase or its regulation. They grew well in the presence of NH_4NO_3, presumably because of their failure to accumulate the toxic nitrite.

3.2. Gibberellin production

Nitrate reduction mutants were similar to the wild type in growth and gibberellin production with NH_4Cl as the nitrogen source (Fig. 2, top). In the first few days of cultivation the dry mycelial mass increased rapidly through balanced growth; after exhaustion of the nitrogen source, the mycelial mass increased more slowly through the assimilation of the carbon source [8]. The mutants grew worse than the wild type with NH_4NO_3 (Fig. 2, center left), as expected, since they could not utilize the nitrate moiety. The production of gibberellins in the presence of NH_4NO_3 was drastically decreased (Fig. 2, center right), more than could be explained by the diminution in mycelial mass. The double mutant SG151 produced less gibberellins than single mutants; this excludes that nitrate acts through a slow conversion to ammonium in leaky mutants. We suspected that nitrate itself inhibits gibberellin production. This suspicion was verified by the addition of nitrate to cultures that could not utilise it (Fig. 2, bottom right). In the wild-type nitrate, as expected, increased the mycelial mass (Fig. 2, bottom left) and delayed gibberellin production for 2–3 days (Fig. 2, bottom right); exhaustion of nitrate and the products of its assimilation led to an increased rate of gibberellin production by the larger mycelial mass.

The inhibition of gibberellin production by nitrate was clearly observed when the nitrate reduction mutants were grown in media that contained different concentrations of NH_4Cl and $NaNO_3$ (Fig. 3). Although nitrate could not be metabolized by the mutants, it decreased the gibberellin production rate. The inhibitory threshold varied from 2 to 10 mM, approximately, depending on the initial concentration of NH_4Cl, which determines the total mass and physiological condition of the mycelia.

3.3. Inhibition of gibberellin production by tungstate

Tungstate (Na_2WO_4), a potent inhibitor of nitrate reductase in cyanobacteria [19], slows down mycelial growth in *Gibbe-*

Table 1
Nitrogen sources utilized by the mutants

Strain	$NaNO_3$	$NaNO_2$	Hypoxanthin	Asparagine	NH_4Cl
IMI58289	+	+	+	+	+
Nitrate reduction mutants					
SG140	−	+	+	+	+
SG141	−	+	+	+	+
SG142	−	+	+	+	+
Nitrite reduction mutants					
SG143	−	−	+	+	+
SG144	−	−	+	+	+
Molybdenum cofactor mutant					
SG147	−	+	−	+	+
Asparagine requiring mutants					
SG146	−	−	−	+	−
SG150	−	−	−	+	−
Nitrite hypersensitive mutant					
SG149	+/−	−	+	+	+
Nitrate and nitrite reduction double mutants					
SG151	−	−	+	+	+
SG152	−	−	+	+	+

Mutants SG140 through SG150 were isolated from the wild-type IMI58289 because of their inability to utilize nitrate as nitrogen source. Mutants SG151 and SG152 were isolated from SG140 because of their inability to utilize nitrite. Normal growth is indicated by +, no growth or residual growth by −.

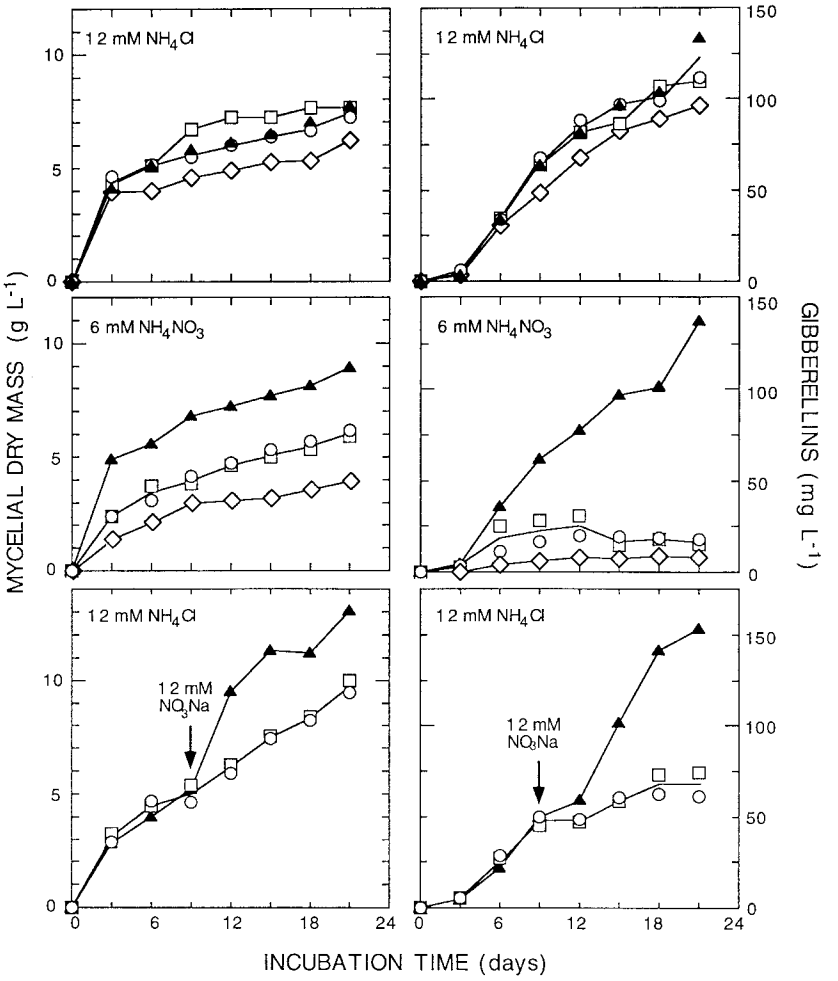

Fig. 2. Effect of nitrate on growth and gibberellin concentrations. The wild type (triangles), the nitrate reduction mutants SG140 (squares), and SG141 (circles), and the nitrate and nitrite reduction double mutant SG151 (diamonds) were cultured in minimal broth with the nitrogen source indicated in each panel. The arrow indicates the addition of $NaNO_3$ at a final concentration of 12 mM.

rella (Fig. 4, top). An unspecific toxic effect occurs at high tungstate concentrations, whether the nitrogen source is ammonium or nitrate; at 30 μM or 100 μM, Na_2WO_4 inhibits growth only when the source of nitrogen is nitrate. Na_2WO_4 100 μM has an effect on gibberellin biosynthesis when the nitrogen source is nitrate (Fig. 4, middle), but not when it is ammonium (Fig. 4, middle). The parameters of tungstate action depend on the molybdenum concentration of the medium; ours contained 0.16 μM $(NH_4)_6Mo_7O_{24}$.

4. Discussion

Wild-type *G. fujikuroi* converts nitrate to ammonium, so that any inhibition of gibberellin biosynthesis by nitrate would be hard to distinguish from the strong inhibition by ammonium, caused presumably by an unknown ammonium metabolite. Mutants that cannot convert nitrate to ammonium have allowed the demonstration of inhibition by nitrate itself. Incubation of the wild type with tungstate, an inhibitor of nitrate reductase, supports the conclusions obtained with the mutants.

The inhibition of gibberellin biosynthesis by nitrate is unexpected, because it is difficult to imagine conditions under which nitrate would not be converted to ammonium and metabolized further, but significant, because the nitrate inhibitory threshold (2 to 10 mM, depending on mycelial mass) is comparable with that of other nitrogen sources (i.e. 5 mM for glycine, [20]). Perhaps the regulation of gibberellin biosynthesis by nitrogen availability was evolutionarily derived from a preexisting mechanism that responded to both ammonium and nitrate.

Nitrate induces and ammonium (or one of its metabolites) represses the production of nitrate reductase in many organisms [21]. A similar situation is suggested by our observation of the antagonism of nitrate and ammonium on nitrate reductase activity.

Tungstate inhibits growth of organisms engaged in nitrate reduction by competing with the molybdenum that plays an essential role in this activity [19,22]. The results with *Gibberella* remind of those reported for *Chlorella* [19], but the threshold is higher and growth does not cease completely even at high concentrations. The inhibition of gibberellin synthesis by nitrate offers a good explanation for the decreased gibberellin production by the wild type in the presence of tungstate.

Mutants in nitrate assimilation are usually isolated after

selection for resistance to chlorate. We screened directly for them, without preselection. The phenotypes of our mutants were generally similar to those described in other Ascomycetes, including *Gibberella* strains that produce no gibberellins [18]. Our collection contained mutants that cannot be isolated as chlorate resistant, such as those specific for nitrite reduction, but did not contain mutants of the major nitrogen regulatory gene, *areA*. The direct screening for mutants was facilitated by the high frequencies of induced mutations observed in *G. fujikuroi* [17], a feature that makes *Gibberella* attractive for the mutational analysis of many biological processes.

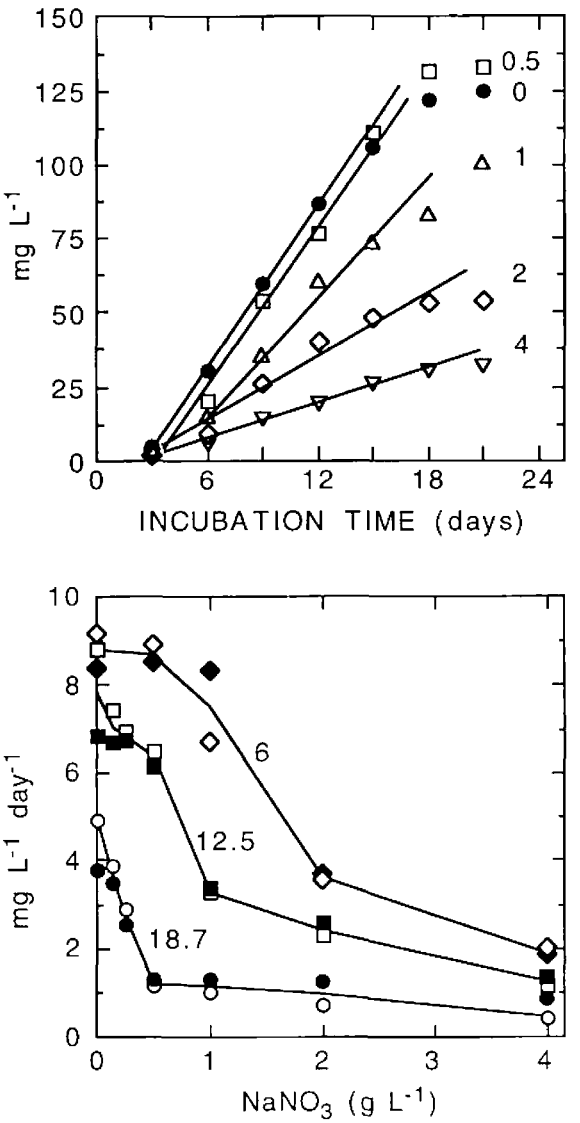

Fig. 3. Inhibition of gibberellin production by nitrate in the nitrate reduction mutant strains SG140 and SG141. Top: An example of the calculation of production rates: gibberellin production by strain SG140 grown in the presence of NH_4Cl (1 g l^{-1}) and $NaNO_3$ (concentrations in g l^{-1} shown on the graphs). The straight lines represent the best linear fit to five consecutive values and their slopes were taken as estimates of the gibberellin production rate. Bottom: Gibberellin production rates by strains SG140 (closed symbols) and SG141 (open symbols) grown in the presence of various concentrations of $NaNO_3$ (abscissae) and NH_4Cl (concentrations in g l^{-1} shown on the graphs).

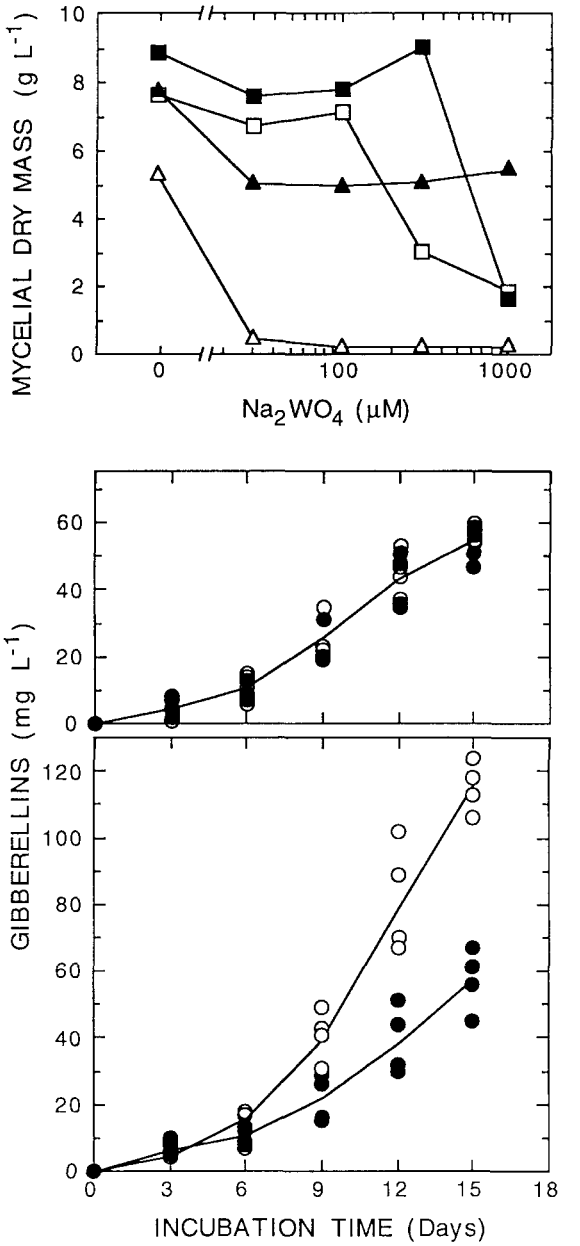

Fig. 4. Inhibition of mycelial growth and gibberellin production by tungstate in the wild type. Top: Mycelial dry mass after incubation for 6 (open symbols) or 15 (closed symbols) days in minimal medium with $NaNO_3$ (triangles) or NH_4Cl (squares) as nitrogen source. Middle and bottom: Gibberellin accumulation in minimal medium with 0.64 g/l NH_4Cl (middle) or 1 g/l NH_4NO_3 (bottom) as nitrogen source in the presence (closed symbols) or the absence (open symbols) of 100 μM Na_2WO_4.

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