EFFECT OF GIBREL ON AZOTOBACTER CHROOCOCCUM

by

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INTRODUCTION

Gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi*, supplements growth promoting substances that are normally found in higher plants, and produces remarkable effects on cell elongation, flowering, and dormancy (19, Vol. 8, p. 181-216).

Few studies concerning its effect on microorganisms have been reported. Lu et al. (13, Vol. 181, p. 189-190) observed that soil treated with 50 ppm gibberellic acid gave unusual growth of *Azotobacter chroococcum* on sodium albuminate agar plates used for total bacterial counts. Further studies by Lu and Bollen (14, Vol. 9, p. 318-324) indicated that 50 ppm Gibrel stimulated nitrogen fixation and hastened decomposition of organic matter in the same soil. It was found that 50 ppm gave maximal effects. Nitrification and sulfur oxidation, functions of autotrophic bacteria, were significantly increased by 50 ppm Gibrel in all but two of eleven widely different Oregon soils studied by Chandra and Bollen (3, Vol. 8, p. 31-38).

The previously published results on stimulation of *Azotobacter* growth and nitrogen fixation by Gibrel in soil

1. Gibrel, $C_{19}H_{21}O_6K$, is the trade-mark registered by Merck Co., Inc., Rahway, New Jersey, for technical grade water-soluble potassium salt of gibberellic acid.

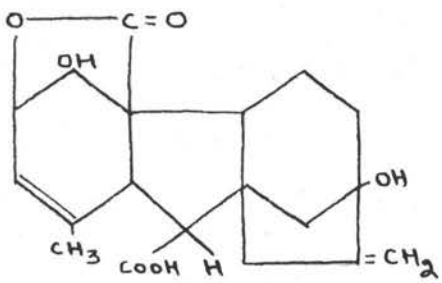
suggested an investigation of its effect on pure cultures of Azotobacter chroococcum. Four different approaches were chosen to attack the problem: (1) optical density measurements; (2) total number of viable cells as shown by plate count; (3) total cell weight or crop yield; (4) Kjeldahl nitrogen determination. Gross stimulatory effects have been observed, but cellular mechanisms were not investigated.

Incidental to the main problem, it became necessary to study the effects of calcium, manganese and phosphate concentrations in order to establish a medium most favorable for growth of the test bacterium.

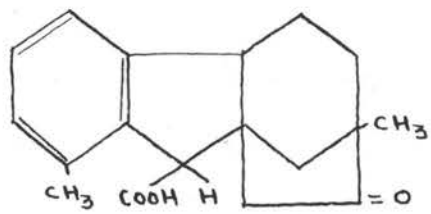
HISTORICAL REVIEW

Gibberellic Acids or the Gibberellins

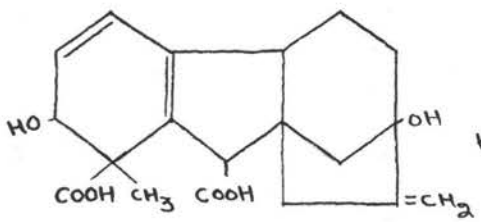
Effects of gibberellic acids or the gibberellins, in higher plants have been studied by many workers. A number of gibberellins have been isolated and their structures determined; among these the following have been most widely investigated.

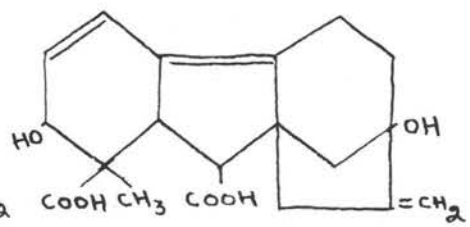


Gibberellic acid



Gibberic acid





Gibberellenic acid

Brian et al. (1, Vol. 5, p. 602-612) reported increased length of stem internodes and of leaves in wheat plants. They also found a decrease in root weight. An increase in cell length of leaf sheath and a decrease in cell width have been shown by Hayashi et al. (8, Vol. 27, p. 672-675).

Brian et al. (1, Vol. 5, p. 602-612) reported no effect on growth of a wide variety of bacteria and fungi. These included the following: Bacillus subtilis, Bacterium aroideae, Bact. Cartovorum, Bact. coli, Bact. phytophthorum, Bact. tracheiphium, Pseudomonas marginalis, Ps. solacearum, Salmonella typhi, Staphylococcus aureus, and Xanthomonas campestris; Absidia glauca, Aspergillus niger, Bortrytis allii, Fusarium coerulem, F. graminearum, Mucor erectus, Myrothecium verrucaria, Penicillium digitatum, P. expansum, P. gladioli, Stemphylium sp, Thamnidium elegans and Trichoderma viridae.

In 1957 Ciferi and Bertossi (4, Vol. 33, p. 114-116) reported no effect of gibberellic acid on yield of cellular material of several yeasts, molds and algae. The organisms studied were Alternaria tenuis, Aspergilus niger, Phytophthora hybernalis, Rhizopus nigricans, Actinomyces albus, Actinomyces griseus, Candida albicans, Sacchromyces ellipsoideus, Chlorella vulgaris, Prototheca portoricensis.

Lu et al. (13, Vol. 181, p. 189-190) reported unusual growth of Azotobacter chroococcum on sodium albuminate agar plates while studying effect of gibberellic acid on plate counts of soil microorganisms. In the same year Lu and Bollen (14, Vol. 9, 318-324) found that Gibrel, a water soluble potassium salt of gibberellic acid increased nitrogen fixation in the same soil. The range of Gibrel

treatment was from 1 ppm to 1000 ppm and the critical range was found to lie within 5 to 50 ppm.

Recently, Chandra and Bollen (3, Vol. 8, p. 31-38) reported that 50 ppm Gibrel effected increases in nitrification of ammonium sulfate ranging from 0 to 69 per cent in different soils, whereas increases in oxidation of added sulfur ranged from 0 to 50 per cent. A general increase in number of molds, bacteria and streptomyces was also found.

Critical Element Essential in Nitrogen Fixation by Azotobacter.

Calcium

Horner and Burk (9, Vol. 48, p. 981-995) studied the calcium, magnesium and iron requirements of *Azotobacter vinelandii* growing with free and fixed nitrogen. With free nitrogen, a minimum of 0.1 to 0.3 millimolal requirement for calcium ion was established for maximum growth. Harris and Gainery (7, Vol. 48, p. 696) while studying respiration of resting *Azotobacter chroococcum* noted that at lower pH levels calcium exerted a marked stimulatory effect upon respiration. Esposito and Wilson (5, Vol. 93, p. 564-567) studied molybdenum, iron and calcium requirements of *Azotobacter* and showed these irons to be specifically required by *A. vinelandii* O when fixing atmospheric nitrogen. Norris and Jensen (16, Vol. 31, p. 198-205) reported that calcium was

essential for the growth of A. vinelandii, A. chroococcum, A. beijerinckii, and A. agile, both in the presence and in the absence of combined nitrogen.

Manganese

Greaves (6, Vol. 36, p. 278) noted stimulation of nitrogen fixation by Azotobacter when manganese, iron and iodine were added to liquid media. Similar results were obtained by Steinberg (18, Vol. 57, p. 461-476) who observed decreased yields of Azotobacter when traces of iron, manganese, molybdenum and calcium were omitted from nutrient solutions with fixed and free nitrogen. However, Jensen (11, Vol. 4, p. 235) while studying magnesium requirements found manganese to be less important than magnesium for general growth of Azotobacter.

Phosphate

In a review of the Azotobacteriaceae, Jensen (12, Vol. 18, p. 200) mentioned the higher requirement for magnesium with inorganic phosphate; only 4 to 8 ppm magnesium were required with calcium glycerophosphate. During a study of the magnesium requirements of Azotobacter and Beijerinckia, Jensen (11, Vol. 4, 224-236) noticed vigorous growth and optimum nitrogen fixation in a medium with either calcium glycerophosphate or 1,6-Fructose-diphosphate.

MATERIALS AND METHODS

Azotobacter chroococcum A.T.C.C. 480 was used throughout the experiment. This was maintained by weekly transfers on mannitol agar slants.

Media

Unless otherwise mentioned, reagent chemicals were used.

Mannitol agar medium for maintenance:

Mannitol	10.0 g
$K_2HPO_4 3H_2O$	0.5 g
$MgSO_4$	0.2 g
NaCl	0.2 g
$FeCl_3, 6H_2O$ (0.1 g/100 ml)	1.0 ml
Molybdic acid (0.133 g/100 ml)	2.3 ml
$MnCl_2 4H_2O$ (0.234 g/100 ml)	2.0 ml
Noble agar (Difco)	15.0 g
Distilled water	1000 ml

Liquid media for study of Gibrel effects:

Basal Medium

Mannitol	10.0 g
$MgSO_4$	0.2 g
NaCl	0.2 g

$FeCl_3,6H_2O$ (0.1 g/100 ml)	1.0 ml
Molybdic acid (0.133 g/100 ml)	2.3 ml
Other constituents - according to modifications	
Distilled water	1000 ml

Inorganic Phosphate Medium

Basal medium+0.5 g $K_2HPO_4,3H_2O$

Organic Phosphate Medium

Basal medium+2.0 g calcium glycerophosphate (N.F. grade) + 0.5 g KCl (crystal).

Manganese Additions

Stock solution of $MnSO_4,H_2O$ (0.2 g/100 ml) and $MnCl_2$, $4H_2O$ (0.234 g/100 ml) were prepared.

For 2 ppm salt ≡ 0.65 ppm Mn^{++} - 1.0 ml/liter of basal medium.

For 4 ppm salt ≡ 1.3 ppm Mn^{++} - 2.0 ml/liter of basal medium.

Unless otherwise stated, manganese was added in the form of $MnCl_2,4H_2O$ at 4 ppm.

Inorganic Phosphate with Glycerol Equivalent to Glycerophosphate

Inorganic phosphate medium + 0.306 ml of glycerol.

Inorganic Phosphate with $CaCl_2$ and Glycerol Equivalent to Calcium Glycerophosphate

Basal medium + 2.168 g $K_2HPO4,3H_2O$ + 1.4 g $CaCl_2$, $2H_2O$ + 0.306 ml of glycerol.

Inorganic Phosphate with $CaSO_4$

(a)	K_2HPO_4	0.8 g
	KH_2PO_4	0.2 g
	$MgSO_4$	0.2 g
	$CaSO_4$ (anhyd)	0.1 g
	Distilled water	1000 ml

After shaking well and standing for 24 hours, the supernatent, was decanted and used for the following medium:

(b)	Clear supernatent (a)	900 ml
	Mannitol	10.0 g
	NaCl	0.2 g
	K_2HPO_4, $3H_2O$	0.2 g
	$MnSO_4$, H_2O(0.2g/100ml)	2.0 ml
	$FeCl_3$, $6H_2O$(0.1g/100ml)	1.0 ml
	Molybdic acid (0.133 g/100 ml)	2.3 ml
	Distilled water	1000 ml

Fructose - 1,6 - Diphosphate Medium

Basal medium	200 ml
KCl	0.1 g

Fructose-1,6-diphosphate 0.4 g

Inorganic Phosphate with 1.5 Times Mannitol

Inorganic phosphate medium with 15 g mannitol, in place of 10 g.

Gibrel, where used, was added in one per cent aqueous solution to sterilized media to give a concentration of 50 ppm. Solutions were either freshly prepared or kept at 10° C not longer than 7 days. Gibrel was used instead of gibberellic acid because the acid is highly insoluble in water.

Medium for Plate Counts

The organic phosphate medium with 1.5 per cent noble agar was used.

Analytical Procedure

Optical density measurements of growth in liquid media was measured after 24 hours using a Bausch and Lomb "Spectronic 20" colorimeter at 625 millimicron wavelength.

Total cell weight or crop yield after 7 days was determined by centrifuging contents of culture flasks at 5000 r.p.m. for 15 to 20 minutes followed by washing. The pellets were quantitatively transferred to aluminum cups, dried at 100° C for 24 hours and weighed.

Nitrogen was estimated by the Kjeldahl procedure, using Hibbard's mixture and selenized granules as catalysts.

Recoveries with standard solutions of ammonium sulfate varied from 99.3 to 101 per cent.

Experimental Procedure

Inocula for shake cultures were prepared from 72 hour old cultures grown on mannitol agar slants at 28° C in 8-ounce prescription bottles. The surface growth was suspended in 10 ml inorganic phosphate liquid medium; 0.7 ml of this suspension was used to inoculate each flask. The cultures were frequently examined for purity by direct microscopic examination of stained slides and by plating.

Shake cultures were grown in 250 ml cotton plugged Erlenmeyer flasks containing 100 ml of liquid medium after sterilizing by autoclaving at 15 p.s.i for 20 minutes. A New Brunswick rotary shaker was used to insure adequate aeration. It was operated at 240 r.p.m., and incubated at 28 ± 3° C.

All treatments were replicated at least twice in each experiment, and each experiment was repeated several times.

RESULTS AND DISCUSSION

Table I illustrates the effect of manganous chloride and manganous sulfate as sources of manganous ion with and without Gibrel. Addition in terms of manganese was 1.3 ppm in each case. The chloride was superior in the organic phosphate medium, which gave better growth than did the medium with inorganic phosphate. The stimulatory influence of Gibrel is evident in each of the media. Inasmuch as the basal medium contained adequate chloride and sulfate ions, nutritional reasons for better growth with manganous chloride are not apparent. However, more precipitate occurred in the medium containing the sulfate.

Table 2 shows the effect of manganese at 0.65 ppm and 1.3 ppm in organic phosphate and inorganic phosphate media, with and without Gibrel. Increased growth at 7 days with Gibrel is well shown by crop yields and plate counts.

Table 3 shows difference between organic and inorganic phosphate in their effects on growth of Azotobacter as determined by optical density, plate counts, crop yields and Kjeldahl nitrogen. Averages of these four criteria indicate the calcium glycerophosphate gave more than four times as much growth as did the inorganic phosphate. This is in agreement with Jensen's (11,Vol. 4, p. 224-236) findings.

Table 4 presents data showing that the better growth

TABLE I

Comparative Effects of Manganous Chloride and Manganous Sulfate on Growth of Azotobacter chroococcum

Medium	Manganese at 1.3 ppm Mn^{++}			
	$MnSO_4$		$MnCl_2$	
	Optical Density*	Crop yield at 7 days Dry basis mg	Optical Density*	Crop yield at 7 days Dry basis mg
Organic phosphate	0.62	60.0	0.64	108.0
Organic phosphate +50 ppm Gibrel	0.66	126.0	0.87	151.0
Inorganic phosphate	0.48	24.0	0.45	26.0
Inorganic phosphate +50 ppm Gibrel	0.51	40.0	0.44	34.8

* Measured at 24 hrs.

TABLE 2

Effect of Manganous Ion Concentration on Growth of *Azotobacter chroococcum*

Medium	Manganese as $MnCl_2$					
	Optical Density*	0.65 ppm Crop yield at 7 days. Dry basis. mg	Plate count at 7 days Millions/ml	Optical Density*	1.3 ppm Crop yield 7 days. Dry basis. mg	Plate Count at 7 days Millions/ml
Organic phosphate	0.71	—	65	0.73	—	60
Organic phosphate +50 ppm Gibrel	0.76	—	260	0.89	—	230
Inorganic phosphate	0.37	26	—	0.43	27	—
Inorganic phosphate +50 ppm Gibrel	0.38	31	—	0.48	35	—

* Measured at 24 hrs.

TABLE 3

Comparative Effects of Organic and Inorganic Phosphate on Growth of *Azotobacter chroococcum*

Medium	Optical Density*	Plate Count at 7 days Millions/ml	Crop yield at 7 days dry basis mg	Kjeldahl Nitrogen flask mg
Organic phosphate	0.57	60	93	10.6
Inorganic phosphate	0.10	10	24	5.8

* Measured at 24 hrs.

obtained with calcium glycerophosphate is due to the molecule as a whole. Combinations of dipotassium phosphate with calcium salts and glycerol gave much less growth than the organic phosphate. Possible ability of the glycerophosphate to act as a hydrogen donor in the nitrogen fixation reaction may account for these findings. Carnahan et al. (2, Vol. 38, p. 188-189) while studying nitrogen fixation in cell free extracts of Clostridium pasteurianum found sodium pyruvate to be a most active substance in the fixation process; ∝-ketobutyric acid and many other hydrogen donors were much less active.

Table 5 shows results of an experiment conducted to determine the effects of different methods of sterilizing the liquid media. Because more or less precipitate always occurred after autoclaving, this interfered with optical density measurements. Attempts to avoid this were made by sterilizing the medium by filtering through Pyrex sintered glass filters UF, and transferring aseptically to sterile flasks. Either the presence of the precipitate favored growth, or filtration removed some essential trace element. Possibly the precipitate retained such elements, or it promoted growth through physical action. It is known that finely divided materials and colloidal substances can increase growth by the greater surface area presented (15, Vol. 4, p. 82-90). Much higher counts were obtained with the autoclaved media, especially with the Gibrel

TABLE 4

Effect of Modified Phosphate Media and Gibrel on growth of *Azotobacter chroococcum*

Medium	Optical Density at 24 hrs	Plate Counts at 7 days Millions/ml	Crop yield at 7 days Dry basis mg
Organic phosphate	0.64	60	99
Organic phosphate 50 ppm Gibrel	0.69	220	133
Inorganic phosphate with glycerol	—	15	39
Inorganic phosphate with glycerol 50 ppm Gibrel	—	180	49
Inorganic phosphate with $CaSO_4$	0.05 x	—	—
Inorganic phosphate with $CaSO_4$ 50 ppm Gibrel	0.08 x	—	—
Inorganic phosphate with $CaCl_2$ and glycerol	*	< 1	—
Fructose-1,6-diphosphate	0.08 x	0	—
Fructose-1,6-diphosphate 50 ppm Gibrel	0.12 x	12	—
Inorganic phosphate with 1.5 times mannitol	0.312	10	26
Inorganic phosphate with 1.5 times mannitol 50 ppm Gibrel	0.36	40	36

x At 7 days
* Not measured; heavy precipitate in medium.

TABLE 5

Comparative Growth of *Azotobacter chroococcum* in Autoclaved and Filter Sterilized Organic and Inorganic Phosphate Media

Medium	Treatment of medium	Plate counts at 7 days Millions/ml	Appearance of medium
Organic phosphate	Autoclaved	5	Turbid
Organic phosphate + 50 ppm Gibrel	"	230	"
Organic phosphate	Filtered	3.5	Clear
Organic phosphate + 50 ppm Gibrel	"	87	"
Inorganic phosphate	Autoclaved	2.5	Turbid
Inorganic phosphate + 50 ppm Gibrel	"	42	"
Inorganic phosphate	Filtered	0.4	Clear
Inorganic phosphate + 50 ppm Gibrel	"	20	"

addition.

Table 6 shows that 50 ppm Gibrel considerably increases plate counts and crop yields of Azotobacter. Data for replicate cultures for two experiments with organic phosphate and one experiment with inorganic phosphate are presented. The Gibrel effect is pronounced in each medium, although more growth was obtained with the organic phosphate. Reasons for the effect of Gibrel are unknown. Recently, however, Ormrod and Williams (17, Vol. 35, p. 81-87) expressed the belief that the stimulatory action of gibberellic and 2,4-dichlorophenoxyacetic acids in plants is closely related to phosphorous metabolism and buildup of growth material reserves.

Table 7 presents data showing that total nitrogen fixed by Azotobacter is definitely increased by the presence of Gibrel at 50 ppm. Again, the superiority of the organic phosphate over the inorganic is demonstrated. Whether the Gibrel increases total nitrogen by influencing the fixation reaction or by increasing mass growth was not determined. However, the data of Table 7 suggest that the increase in cell mass could be the main factor in increasing total nitrogen fixed.

Table 8 is presented to show correlation between intensity of pigmentation and total nitrogen fixed.

TABLE 6

Gibrel Effect on Plate Counts and Crop Yield of *Azotobacter chroococcum* in Organic and in Inorganic Phosphate Media

	Plate count at 7 days Millions/ml Gibrel ppm		Crop yield at 7 day dry basis mg Gibrel ppm	
	0	50	0	50
Organic phosphate I	60	230	103	143
	60	220	119	164
Organic phosphate II	10	190	90	155
	20	190	89	150
Inorganic phosphate	6	110	27	35
	30	150	—	—

TABLE 7

Gibrel Effect on Total Nitrogen Fixed by *Azotobacter chroococcum* in Organic and in Inorganic Phosphate Media

Medium	Total Nitrogen at 7 days mg/flask
Organic phosphate	7.5
"	8.1
Organic phosphate + 50 ppm Gibrel	10.4
"	9.2
Inorganic phosphate	4.8
"	4.1
Inorganic phosphate + 50 ppm Gibrel	5.8
"	5.2

Increased fixation was found to accompany increased pigment production. Highest total nitrogen values were almost always obtained in definitely black cultures while lowest values were always found where color was definitely absent. It may be mentioned that no pigmentation was ever observed with only 0.65 ppm manganese in inorganic phosphate medium.

Microscopic observations revealed no significant effects of Gibrel on morphology of cells stained with methylene blue.

Because crop yield, plate counts and Kjeldahl nitrogen determinations were not proportional this could be due to differences between viable counts and total number of cells, which were not determined microscopically.

The low counts at 7 days compared to Escherichia coli and other rapidly growing bacteria are not unusual for Azotobacter (10, Vol. 65, p. 176) because it has a low growth rate in nitrogen-free synthetic medium.

TABLE 8

Correlation of Pigmentation of *Azotobacter chroococcum* with Total Nitrogen Fixed

	Total nitrogen Fixed at 7 days mg per 100 ml of medium			
	Color			
	Black	Brown	Light brown	Colorless
	9.5	4.4	3.7	1.3
	9.7	5.8	2.5	1.2
	8.6	6.2	4.3	0.8
	10.6	8.1	4.8	2.3
	12.0	7.2	6.4	0.4
	11.4	5.8	5.2	0.5
	9.6	5.9	—	0.5
	7.2	6.0	—	—
	—	6.4	—	—
	—	6.8	—	—
Mean	9.8	6.3	4.5	1.0

SUMMARY

Results of the experiments presented in the foregoing tables are the following:

1. Manganous chloride ($MnCl_2,4H_2O$) was found to be superior to manganous sulfate ($MnSO4\ ,H_2O$) for the general growth of the culture studied, Azotobacter chroococcum A.T.C.C. 480.
2. More growth was observed with 1.3 ppm Mn^{++} compared to 0.65 ppm Mn^{++}. Higher concentrations of Mn^{++} were not used because they increased precipitates in the medium and thus interfered with optical density measurements.
3. Organic phosphate in the form of calcium glycerophosphate as the whole molecule gave much greater growth and more total nitrogen fixed than did inorganic phosphate in the form of the dipotassium salt. The calcium glycerophosphate was superior also to various combinations supplying calcium, carbon and phosphorous equivalent to calcium glycerophosphate.
4. Gibrel exerted stimulatory effects on growth and nitrogen fixed, in all different media used for Azotobacter chroococcum.
5. The significant differences in results obtained with autoclaved and filter sterilized media cannot be directly explained. The better results with autoclaved

media may tentatively be attributed to the colloidal nature of the precipitate and increased surface exposure offered for growth.

6. The total nitrogen fixed by Azotobacter chroococcum was always greatest with maximum pigmentation as shown by definitely black color. In inorganic phosphate media containing only 0.65 ppm Mn^{++} and giving only poor growth of Azotobacter, pigmentation was light or totally absent. This was not true for calcium glycerophosphate media which produced black cultures in all cases.

Studies on how Gibrel acts to give the apparent increases in plate counts, crop yields and total nitrogen fixed and why they are not proportional are underway.

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