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Dinitrogen fixation activity of *Azospirillum brasilense* in maize (*Zea mays*)

S. P. Saikia[1,2,*], Vanita Jain[1], Sangeeta Khetarpal[1] and Samitha Aravind[1]

[1]Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi 110 012, India
[2]Division of Medicinal, Aromatic and Economic Plants, North East Institute of Science and Technology (CSIR), Formerly Regional Research Laboratory, Jorhat 785 006, India

The association of maize with the N_2-fixing bacteria, *Azospirillum* was examined to evaluate possible benefits for agriculture. Maize seedlings develop nodule-like tumour knots (*para*-nodules) along primary roots when treated with the auxin 2,4-dichlorophenoxyacetic acid (2,4-D). The presence of nitrogenase activity (acetylene reduction assay) was noticed in plants treated with *Azospirillum* along with 2,4-D (nodulated plants) at various stages of growth. The 2,4-D induced nodule-like structures provided niche for the establishment of the bacteria, thereby enhancing nitrogen fixation in the nodulated roots. *Azospirillum* inoculation had a significantly positive effect on the activity of the enzymes glutamine synthetase and glutamate synthase in leaves of *para*-nodulated maize plants. The *para*-nodulated plants showed higher rates of net photosynthesis, stomatal conductance leading to higher yield compared to non-nodulated (control) plants. The

*For correspondence. (e-mail: sp_sidhu@yahoo.com)

nutrient content in grains and stover was higher in the case of *para*-nodulated plants. The results provide evidence that maize plants are potentially able to create a symbiosis with diazotrophic bacteria, which colonize *para*-nodule tissue intracellularly, promoting a higher level of N_2 fixation for better growth and development of plants. Further, our data suggest that an increased understanding of plant root–rhizosphere N_2-fixing bacterial interactions is required before any consistent, significant and beneficial N_2-fixing association can be developed.

Keywords: Acetylene reduction, *Azospirillum brasilense*, maize, physiological activities, seedling inoculation.

The times have changed; the face of the country is changed; the quality of the soil has changed; and if we will live as well, and become as rich and respectable as our fathers, we must cultivate their virtues; but abandon their farming system.

The Farmers Manual (1819)

BIOLOGICAL nitrogen fixation is the key to sustain agricultural productivity through the application of biofertilizers in the field. Increased foodgrain cannot be produced unless we carefully make use of biological nitrogen fixation. Since the nodulation per se is a physiological process, it is possible to induce nodulation using different plant growth regulators and it is obvious that the nitrogen-fixing bacteria can enter through cracked epidermal cells of the nodules and such nodules can provide a niche for the bacteria that fixes nitrogen inside the nodules[1]. Among different plant hormones, 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, is the best in inducing nodular outgrowth in cereals[2]. The objectives of this study were to determine the amount of N_2-fixation by *Azospirillum* in association with *Zea mays*, and to measure whether inoculation with this organism established an association that could contribute significant amounts of fixed N to plants.

Seeds of *Z. mays* L., cultivar Kiran were surface-sterilized and germinated on sterile, acid-washed gravel culture at 25°C for 5–6 days. Uncontaminated seedlings from the acid-washed gravel culture were grown in glass tubes (20 cm length, 3 cm diameter, one seedling per tube) containing sterile N-free Hoagland solution at 25°C in growth room. Exposure of the root zone to light was prevented. Bacterial inoculations were performed two days after transferring the seedlings to Hoagland solution with 0.1 ml of *Azospirillum brasilense* (Sp-29, culture collection, Division of Microbiology, IARI, New Delhi) culture grown at room temperature for 24 h, containing 10^7–10^8 cells/ml along with 1.0 ppm 2,4-D. Nodule-like structure were formed on the roots after a period of 7–10 days after treatment with 2,4-D. The two-week-old seedlings after nodulation under laboratory conditions were transferred to pots (50 cm × 50 cm × 50 cm) in the net house under natural conditions. Each pot was filled with 45 kg of soil. After transplanting in pots, biofertilizer *Azospirillum* was applied in the form of solution 20 ml/pot @ 10^7–10^8 cells/ml. No chemical fertilizers were applied. Nitrogenase activity was assayed in actively growing intact nodulated roots at 30, 60 and 90 days after transplanting (DAT) in pots following the acetylene reduction assay (ARA) method[3], with slight modifications: 5 cc of gas or air was removed and 5 cc of acetylene was injected into the vials containing 1 g of nodulated roots and incubated for 12 h at 30°C. Gas mixture (2 cc) was fed to the gas chromatograph and the acetylene reduced was measured quantitatively. Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities were assayed in actively growing leaves of *para*-nodulated plants at 30, 60 and 90 DAT. The activities of GS and GOGAT were estimated using the spectrophotometric method[4]. Two grams of leaves was ground in extraction buffer, 100 mM Tris buffer (pH 7.5). The homogenate was filtered through a cheese cloth and centrifuged at 10,000 rpm at 4°C for 15 min. The assay for GS was carried out at 37°C (Reaction mixture consisted of Tris, 100 μmol; $MgSO_4$, 100 μmol; cysteine, 10 μmol; L-glutamate, 250 μmol; ATP, 10 μmol; enzyme extract, 0.1–0.5 ml, and hydroxylamine, 20 μmol in the reaction volume of 3 ml). The reaction was terminated by adding ferric chloride reagent

Table 1. Nitrogenase activity (nmol of ethylene/g fr. wt/h) in roots of control and *para*-nodulated maize plants

	Days after transplanting		
Treatment	30	60	90
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Nodulated	1.13* ± 0.34	2.68* ± 0.36	2.86* ± 0.42

*Values were significant at 5% level of probability.

Table 2. Glutamine synthetase activity (μmol/g fr. wt/h) in leaves of control and *para*-nodulated maize plants

		Days after transplanting		
Treatment		30	60	90
Control	Leaf	89.57 ± 0.42	148.80 ± 1.40	139.33 ± 3.64
Nodulated	Leaf	102.64* ± 1.73	188.27* ± 1.43	167.59* ± 2.48

*Values were significant at 5% level of probability.

Table 3. Glutamate synthase activity (μmol NADH oxi/min/g fr. wt) in leaves of control and *para*-nodulated maize plants

		Days after transplanting		
Treatment		30	60	90
Control	Leaf	37.87 ± 1.09	100.80 ± 0.00	55.95 ± 0.00
Nodulated	Leaf	25.11 ± 1.03	132.59* ± 3.12	84.78* ± 1.10

*Values were significant at 5% level of probability.

Table 4. Photosynthetic rate (μmole CO_2/sq. m/S) and stomatal conductance (m/S) in control and *para*-nodulated maize plants

	Treatment			
	Photosynthetic rate		Stomatal conductance	
Days after transplantating	Control	Nodulated	Control	Nodulated
30	3.70 ± 0.38	11.17* ± 0.06	0.06 ± 0.01	0.12* ± 0.06
60	5.40 ± 0.77	12.98* ± 0.18	0.16 ± 0.05	0.32* ± 0.08
90	6.90 ± 0.20	14.28* ± 0.13	0.22 ± 0.03	0.36* ± 0.07

*Values were significant at 5% level of probability.

Table 5. Fresh and dry weight of leaf (g/plant) and leaf area (sq. cm) in control and *para*-nodulated maize plants

		Leaf		
Days after transplanting	Treatment	Fresh weight	Dry weight	Leaf area
30	Control	23.99 ± 1.94	5.90 ± 0.32	1527.84 ± 41.91
	Nodulated	42.63* ± 2.34	7.86* ± 0.51	1647.89* ± 89.45
60	Control	35.96 ± 0.41	7.01 ± 0.17	1838.24 ± 31.50
	Nodulated	46.43* ± 0.21	9.49* ± 0.13	2187.21* ± 64.04
90	Control	20.65 ± 1.54	6.85 ± 0.20	1286.83 ± 47.97
	Nodulated	32.73* ± 0.65	11.54* ± 0.87	1562.29* ± 40.34

*Values were significant at 5% level of probability.

Table 6. Fresh and dry weight of stem (g/plant) in control and *para*-nodulated maize plants

		Stem	
Days after transplanting	Treatment	Fresh weight	Dry weight
30	Control	34.88 ± 0.39	3.14 ± 0.04
	Nodulated	44.53* ± 2.57	7.40* ± 0.55
60	Control	75.99 ± 1.72	15.33 ± 0.58
	Nodulated	101.22* ± 5.71	22.80* ± 1.83
90	Control	49.94 ± 1.68	17.42 ± 0.93
	Nodulated	112.57* ± 2.57	39.70* ± 1.92

*Values were significant at 5% level of probability.

Table 7. Yield and yield components in *para*-nodulated maize at final harvest

Treatment	Ear length (cm)	No. of grains per ear	Hundred grain weight (g)
Control	13.33 ± 0.08	248.67 ± 1.02	22.07 ± 0.02
Nodulated	16.50* ± 0.09	329.67* ± 2.03	32.39* ± 0.02

*Values were significant at 5% level of probability.

and absorbance taken at 540 nm. The reaction mixture for GOGAT contained Tris, 75 μmol, α-ketoglutaric acid, 10 μmol; L-glutamine, 15 μmol, NADH, 0.3 μmol, and enzyme aliquot, 100 μL in the total reaction volume of 3 ml. Readings were taken at 340 nm (UV-Vis spectrophotometer, EC).

The fresh and dry weight of the stem and leaf and leaf area was measured at 30, 60 and 90 DAT. The stem dry weight was measured after drying the material for 72 h at 70°C. The leaf dry weight was determined after drying the leaves to a constant weight and leaf area was measured using a leaf area meter (LI-3100, LI-COR, Inc., Lincoln, NE, USA). Photosynthetic rate and stomatal conductance were measured with the help of an Infra Red Gas Analyser (LI-COR 6200). The yield components as well as the nitrogen, phosphorus and potassium content in stover and grain were estimated at final harvest. Dried plant samples were ground to a fine powder and used for the estimation of nutrients. Nitrogen was estimated using an auto analyser (Technicon, USA). Phosphorus was estimated using standard procedure[5] and potassium in a flame photometer (model CL 22D). For estimation of phosphorus and potassium, samples were digested with the diacid mixture. The experiment was laid out in completely randomized design. All the observations are the means of three replicates and comparisons of treatment means were made at the 5% confidence level[6].

The 2,4-D treatment (1.0 ppm) induced the nodule-like tumour knots (*para*-nodules) in maize roots. It started from the steler portion, and cell division started at the inner cortex level and pushed the epidermal cells. These cells bulge out in the shape of a nodule. The inoculated bacteria *Azospirillum* entered through loosening of the epidermis without causing much damage to infected host epidermal cells[7,8]. Acetylene reduction rate was observed in nodulated plants inoculated with *Azospirillum brasilense* at different stages of growth. Control seedlings showed no acetylene reduction (Table 1). Nitrogenase ac-

Table 8. NPK content (%) in grain and stover in *para*-nodulated maize at final harvest

	N content		P content		K content	
Treatment	Grain	Stover	Grain	Stover	Grain	Stover
Control	1.39 ± 0.08	0.94 ± 0.04	0.11 ± 0.02	0.09 ± 0.04	0.10 ± 0.02	0.28 ± 0.06
Nodulated	1.52* ± 0.09	1.24* ± 0.06	0.13* ± 0.03	0.11* ± 0.02	0.11* ± 0.02	0.30* ± 0.07

*Values were significant at 5% level of probability.

tivity of *A. brasilense*, in terms of associative symbiotic nitrogen fixation, has been reported by many workers[9,10]. The 2,4-D induced nodule-like structure serve as a niche for bacterial colonization in maize roots, thereby enhancing the nitrogen-fixing capacity in the roots. Enhanced nitrogenase activity observed in maize *para*-nodules might be due to the efficiency of the host to encapsulate the intracellular colonizing bacterial cells with a membrane layer resembling the peri-bacteroid membrane of the legume nodule[2]. This also indicates that the host plant was capable of supplying the necessary substrate for N_2-fixation (C_2H_2 reduction) of the bacteria residing mainly in the *para*-nodule. Biological nitrogen fixation during nodular association or *nif* gene transfer to the plants is high[11]. The bacteria better express their N_2-fixing potential inside nodular tissues due to lower competition for nutrients and protection against high level of O_2 pressure on the root surface[12]. Demonstration of significant N_2-fixation in maize seedling roots using 2,4-D and *Azospirillum* is an important development, which may open new fields for both basic and applied aspects of N_2-fixation.

Plants treated with *Azospirillum* along with 2,4-D (nodulated plants) showed increased activity of GS and GOGAT in leaves (Tables 2 and 3), and increased photosynthetic rate and stomatal conductance when compared with uninoculated controls at different stages of growth (Table 4). These results are in accordance with the earlier findings reported in wheat plants[13]. The fresh and dry weight of leaf and stem (Tables 5 and 6) were also found to be higher in the nodulated plants at different stages of growth. The higher biomass production in nodulated plants may be due to higher leaf area (Table 5) and better root and shoot growth, which tends to absorb more water and nutrients from the deeper zone of the soil combined with the better nitrogen fixation capacity of the *Azospirillum*-treated nodulated plants[14].

Data pertaining to the yield parameters (Table 7) showed that inoculation with *A. brasilense* along with 2,4-D produced higher number of ears per plant with increased ear length, ear weight and higher number of grains per ear. Yield increases obtained in inoculated plants, however, have been attributed to biological nitrogen fixation and may also be due to the production of growth substance by the colonizing bacteria. Increased N supply through N_2 fixation, higher nutrient uptake[15] and higher photosynthetic rate and stomatal conductance in inoculated plants might have contributed to the increase in grain yield.

In the present experiment no chemical source of N was added and the significant increase in the level of nutrients was obtained. The major nutrients, viz. nitrogen, phosphorus and potassium in grains and stover increased significantly in *para*-nodulated maize plants compared to control (Table 8). Data on NPK content in grains and stover at final harvest in plants revealed that while nitrogen and phosphorus content were higher in grains, potassium was high in stover. Increase in nitrogen content in shoot and grains has been reported in cereal[16]. Increased uptake of mineral ion by maize and sorghum has been reported under laboratory conditions[17].

Proper understanding of plant bacterial associations is necessary if we are to increase the N nutrient input by rhizosphere bacteria and improve the N nutrition of cereal crops by this method. The population dynamics of introduced *A. brasilense* inside *para*-nodules must be studied during the entire period of plant growth and the overall N gain of the symbiotic system must be investigated by N-balance calculations or with ^{15}N-isotope techniques. Studies of associative symbiosis should be pursued vigorously because of their potential importance, and because the plant and bacterial association examined to date has been limited.

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Enhancement of cytotoxicity and DNA binding of cisplatin in Dalton's lymphoma cells by α-tocopherol

Suniti Sarna* and Rakesh K. Bhola

Department of Zoology, Gauhati University, Guwahati 781 014, India

A combination treatment of cisplatin and α-tocopherol on Dalton's lymphoma cells *in vitro* was studied in order to examine the effect of α-tocopherol on cisplatin cytotoxicity and binding of platinum to DNA (DNA platination) or its removal from DNA. Cisplatin cytotoxicity which is measured as cell survival of Dalton's lymphoma cells was found to be enhanced by various factors, including concentration of drug, treatment duration and presence of α-tocopherol in the medium. Tumour cells treated with increasing concentrations of cisplatin or cisplatin with α-tocopherol lower cell survival and increase DNA platination. There was a significant correlation between percentage survival of Dalton's lymphoma cells and DNA platination. The study of percentage removal of platinum has shown an inverse linear correlation between DNA damage and DNA repair. The enhanced cytotoxicity of cisplatin by α-tocopherol could be attributed to increased platinum binding to DNA, which might decrease DNA repair.

Keywords: Cisplatin, cytotoxicity, Dalton's lymphoma, DNA platination, α-tocopherol.

CISPLATIN is successfully used in the chemotherapy of a wide variety of experimental malignant tumours[1–3] and also in the treatment of many human cancers[4,5]. Many reports have been published describing the intercalation of cisplatin with DNA[6–8], which suggest that cellular DNA could be the primary target for cisplatin in its cytotoxicity. The antitumour activity of cisplatin is believed to result from the interaction of drug with DNA, which leads to the formation of different types of adducts through reaction of the bifunctional platinum compound with N^7 atoms of the nucleobases guanine and adenine. The major adducts are intrastrand crosslinks formed by the binding of cisplatin on two neighbouring guanines, on adenine and guanine and on two guanines separated by one or more nucleobases. Other types of adducts formed are the interstrand crosslinks on two guanines and monofunctionally bound cisplatin on guanines[8–10]. Hyperthermia enhances the cytotoxic effect of cisplatin in both cisplatin-sensitive and cisplatin-resistant mammalian cell lines[11–13]. The enhanced cytotoxicity may be due to increased DNA crosslinking by cisplatin, increased cellular accumulation of platinum or decreased repair of drug-induced DNA damage[12–14]. The amount of platinum bound to DNA is also likely to be related to the sensitivity of cells to cisplatin[15]. Prasad and Rama[16] showed that vitamin E induced both morphological differentiation and growth inhibition of murine and human neuroblastoma cells in culture at least in part by an antioxidant mechanism. Vitamin E probably inhibits DNA synthesis in tumour cells because it inhibits cell division. It may act similarly at the site on RNA or DNA where cisplatin binds[17]. Prasad *et al.*[17] demonstrated the presence of vitamin E-binding proteins in tumour cells in culture, but the relationship of vitamin E-binding proteins to the mechanism of action of vitamin E is unknown. The association of a significant amount of radioactive vitamin E with the purified chromatin suggests that vitamin E may modulate genetic expression in mammalian cells[17]. The present study was undertaken to investigate the effects of vitamin E on cisplatin cytotoxicity and on binding of platinum to DNA (DNA platination) or its removal from DNA.

*For correspondence. (e-mail: sunitisarna@yahoo.com)