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Effect of 2,4-D treatment and *Azospirillum* inoculation on growth of *Cymbopogon winterianus*

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The association of *Cymbopogon winterianus* (Citronella), a member of the Poaceae family, with the N_2-fixing bacteria, *Azospirillum* was examined to evaluate possible benefits for agriculture. The nodules on citronella were induced using plant growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D) 1.0 ppm in nitrogen free Hoagland's solution. Concentration of 2,4-D greater than 1.0 ppm caused stunning and death, while concentration of 2,4-D less than 1.0 ppm has no obvious effect on seedling growth. The presence of nitrogenase activity (acetylene reduction assay) and leghemoglobin (hemoprotein found in the nitrogen-fixing root nodules) was noticed in the plants treated with *Azospirillum* either alone or with nodulated plants, but higher activity was noticed in nodulated *Azospirillum* treated plants. These results confirmed the nitrogenase activity of *Azospirillum brasilense* in association with 2,4-D induced nodules of citronella. After transplanting to pots, it was noticed that nodulated and *Azospirillum* - treated plants showed higher chlorophyll content in leaf, enhanced nitrate reductase (NR) activity, leading to higher yield as compared to the control plants (non-nodulated). The nodulated plants treated with *Azospirillum* also had higher physiological activities as compared to plants treated only with *Azospirillum*. The results provide evidence that the plants belonging to the family Poaceae are potentially able to create a symbiosis with diazotropic bacteria, which colonize *para*-nodule tissue intracellularly, promoting a higher level of nitrogen (N_2) fixation leading to better growth and plant development.

Key words: Cymbopogon, nodulation, 2,4-D, *Azospirillum brasilense,* nitrogen fixation, oil yield.

INTRODUCTION

Increasing of fertilizer costs and increasing demand for food have emphasized for full exploitation of biological nitrogen fixation. While the attempts to transfer *nif* gene to plants to make them diazotrophic have not been successful, attempts are afoot to induce symbiotic nitrogen fixation in non-nodulating plants. Excess nitrogen in the global system in its various forms augments green house effects, diminishes ozone layer, promote smog, contaminates drinking water, acidifies rain and stresses ecosystems (Socolow, 1999). Maximum uptake of nitrogen fertilizer is 40-50% of the total applied N_2. Most of the remainder is lost by denitrification and leaching. There are several reports on induction of nodule like structure termed as *para*-nodules, on cereal

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Abbreviations: NAA, Naphthalene acetic acid; **BAP,** benzylaminopurine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **$HgCl_2$,** mercuric chloride; **DAT,** days after transplantation; **DMSO,** dimethyl sulfoxide; **NEDD,** N (1-naphthyl) ethylenediamine dihydrochloride; **BNF,** bacterial nitrogen fixation; **N_2,** nitrogen; **NR,** Nitrate reductase; **FYM,** farm yard manure.

roots by using different plant hormones like 2,4-D, NAA, BAP and Zeatin. Wilde (1951) observed that the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D, a powerful synthetic auxin) induced nodule-like outgrowths on the root of beans. Arora et al. (1959) found that kinetin induced pseudo nodules on tobacco roots. There is also evidence that nodule-like outgrowths can be induced on tobacco roots by alteration in nutrient medium (Tryon, 1955). The term *p*-nodule (*para*-nodule) was introduced by Tcahn and Kennedy to describe the chemically induced nodule since it differs from the naturally occurring legume nodule (Kennedy et al., 1990), which lead them to consider their potential use in constructing N_2-fixing systems with plants. This induced nodule like outgrowths are modified lateral roots with carbon reserves (as starch in amyloplasts) similar to those found in cortex of roots and the microorganisms are able to modulate or interfere with the development of these outgrowth (Ridge et al., 1992). Interestingly these *para*-nodules have been shown to be colonized by diazotrophic bacteria *Azospirillum* which fix nitrogen within these *para*-nodules. Among different plant hormones, 2,4-D a synthetic auxin, was found to be the best in inducing nodular outgrowth in cereals (Ridge et al., 1992; Christiansen-Weniger and Vanderleyden, 1994).

In the present study, rooted slits of *Cymbopogon winterianus* Jowitt. were inoculated with the *Azospirillum brasilense* and nodules were induced by treating the roots with 2,4-D under hydroponical conditions. The symbiotic relationship between plants and bacteria was analyzed in terms of nitrogen fixation, growth and yield patterns of the transplanted citronella in the pots after nodule induction under laboratory conditions. The BNF provides economic and environmental advantages, being characterized as an important tool in achieving a more sustainable crop production. Exploitation of BNF in the plants belonging to the family Poaceae (grass) is a recent possibility, with relatively low efficiency, however, the optimization of the processes can bring significant benefits, since plants of this family are of paramount importance in producing food, fibre and energy (Barbora et al., 2012).

MATERIALS AND METHODS

Bacterial culture, plant host and induction of *p*-nodule

Rooted slips of *Cymbopogon* variety Jor-Lab C-2, developed by CSIR-North East Institute of Science and Technology, Jorhat, Assam, India were surface sterilized using 0.5% $HgCl_2$ for 2 min and rinsed thoroughly with distilled water. Uncontaminated seedlings were grown *in vitro* in glass tubes (length 20 cm, diameter 3 cm, one slip per tube) containing sterile nitrogen free Hoagland solution at 25°C under continuous lighting (600 $\mu Em^{-2}s^{-1}$). The glass tubes were covered with black paper to avoid the exposure of root zone to light. Bacterial inoculation was performed two days after transferring the seedlings to glass tubes with 0.1 ml *A. brasilense* (Sp7, wild-type strain) culture grown at room temperature for 24 h, containing 10^7 to 10^8 cells/ml and a sufficient quantity of filter sterile 2,4-D solution were added to a final concentration 1.0 ppm (2,4-D).

Nitrogen fixation (C_2H_2 reduction)

Nitrogenase activity was assayed in actively growing intact nodules with roots after two weeks. Rooted slips of *Cymbopogon* were removed from the glass tube, washed carefully with the sterile dimineralized water, and transferred to glass vials closed air tight with rubber seal. The nitrogenase activity was estimated following the acetylene reduction method (Hardy et al., 1968) with slight modification: 5cc of gas or air was removed and 5cc of acetylene (C_2H_2) was injected into the vials containing 1 g of nodulated roots and incubated for 12 h at 30°C. Gas mixture (2cc) was fed to the gas chromatograph (Hewlett-Packard 5890, flame ionization detector) and the acetylene reduced was measured quantitatively. The leghemoglobin content was also estimated using modified procedure of Hartree (1955). Since the individual nodules were very small, nodulated roots were taken from each treatment in three replicates. About 200 mg of nodulated roots were homogenized in 4 ml of chilled 0.1 M phosphate buffer (pH 7.0) and the extract was centrifuged for 20 min at 4°C. 2 ml of the supernatant along with 2 ml of 0.2 N NaOH was taken in test tubes. After 30 min 2 ml of pyridine and 100 mg of $Na_2S_2O_4$ was added and kept for one hour at room temperature. The absorbance of the aliquot after centrifugation was read at 555 nm using Spectronic 20.

Growth and yield parameters

The two week old seedlings after nodulation under laboratory conditions were transferred to pots (50 × 50 × 50 cm^3) in a net house under natural conditions. Each pot was filled with 45 kg of soil and well decomposed FYM was added at the rate of 2 kg per pot. Three healthy seedlings were maintained in each pot in a replicated trial and the growth parameters were measured at 30 and 60 DAT. The chlorophyll content in the leaves was estimated using DMSO (Hiscox and Isrealstam, 1979). The tubes containing leaf tissues and DMSO were kept in hot air oven adjusted at 65°C for about 3 h. The absorbance of the chlorophyll extract was measured at 663 and 645 nm using Spectronic 20. The nitrate reductase activity was measured in the leaves using the method of Klepper et al. (1971). The first fully expanded leaf from the top was used for assaying the nitrate reductase activity. 0.3 g of leaf material were put into ice cold infiltration tubes containing 2.5 ml of KNO_3 and 2.5 ml of phosphate buffer (pH 7.5). The tubes were then passed through a vacuum for 5 min so that the nitrate infiltrated the leaf tissue. These were incubated at 33°C for 1 h in the dark. For the assay of enzyme activity, 0.2 ml of reaction mixture from each tube was transferred to another set of test tubes containing 1.0 ml of sulfanylamide and 1.0 ml of NEDD. The absorbance of reaction mixture was measured at 540 nm using a Spectronic 20 instrument. Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities were assayed in actively growing leaves of *para*-nodulated plants at 30 and 60 DAT.

The activities of GS and GOGAT were estimated using the spectrophotometric method (Mohanty and Fletcher, 1980). Two grams of dried 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; MgSO4, 100 µmol; cysteine, 10 µmol; L-glutamate, 250 µmol; ATP, 10 µmol; enzyme extract, 0.1-0.5 ml; and hydroxyl-lamine, 20 µmol in the reaction volume of 3 ml). The reaction was terminated by adding ferric chloride reagent 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). Photosynthetic rate and stomatal conductance were measured using Portable Photosynthesis System, TPS-2 (PP Systems). The

Table 1. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on nitrogenase activity and leghemoglobin content in seedling roots of citronella.

Treatment	Nitrogenase Activity (n moles of ethylene/g F.W./h)	Leghaemoglobin Content (µg/g F.W.)
Control	0.000	0.000
2,4-D	0.000	0.000
Azospirillum	0.112*	0.022*
2,4-D+ *Azospirillum*	0.253*	0.053*

*Values were significant at 5% level of probability

yield components were measured at 60 DAT and also the nitrogen content of the stem, leaf and root at 60 DAT was estimated by using an auto analyzer (Technicon, USA).

Statistical analysis

The experiment was laid out in completely randomized design (CRD). All the observations are the means of three replicates and comparisons of treatment means were made at the 5% confidence level (Panse and Sukhatme, 1967).

RESULTS AND DISCUSSION

Effect of 2,4-D and inoculation with *Azospirillum brasilense* on nitrogenase activity and leghemoglobin

When treated with 2,4-D (1.0 ppm) citronella plants developed nodule like knots along primary roots and club shaped tumours at their root tips after a period of 7-10 days compared with untreated plants. However, well developed nodule like tumour knots that can best be described as modified lateral roots (*para*-nodules) emerged only when the plants were inoculated with *Azospirillum* along with 2,4-D. With the induction of *para*-nodules, poaceous plants produce a specific root tissue, which form a suitable colonization niche for ecologically disadvantaged *A. brasilense.* This plant-bacteria relationship meets essential prerequisites, which demonstrate that it is a true symbiosis (Christiansen-Weniger C and Vanderleyden J, 1994). The nitrogenase activity of *A. brasilense,* in terms of associative symbiotic nitrogen fixation, has been reported by many workers (Okon, 1985; Tilak and Subba Rao, 1987; Rodriguez et al., 2004; Cassan et al., 2009; Couillerot et al., 2012). The acetylene reduction rate was found to be sufficiently high with *Azospirillum* along with 2,4-D than with *Azospirillum* alone (Table 1). Both control and only 2,4-D treated seedlings showed no acetylene reduction (Table 1). The 2,4-D induced nodule like structures therefore might serve as niche for bacterial colonization in citronella roots thereby enhancing the nitrogen fixing capacity in the roots. Enhanced nitrogenase activity observed in citronella *para*-nodules inoculated with *A. brasilense* along with 2,4-D might be due to the efficiency of the host to encapsulate the intracellularly colonizing bacterial cells with a membrane layer, resembling the peribacteroid membrane of the legume nodule (Christiansen-Weniger, 1997). Non-nodular BNF is likely to have very low potential in terms of the amount of N_2 fixed relative to the nodular association or *nif* gene transfer to the plant (Dawe, 2000). 2,4-D increases the specific activity of nitrogenase and the amount of *Azospirillum* bound, thus increasing the N_2- fixing potential inside the nodule tissue due to lower competition for nutrients and protection against high level of O_2 pressure over the root surface (Boddey and Dobereiner, 1995). Thus, indicate 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 *p*-nodule. The demonstration of significant N_2-fixation in the citronella seedling roots using *A. brasilense* along with 2,4-D is an important development, which may open new fields for both basic and applied aspects of N_2-fixation.

Presence of leghemoglobin was found in the treatments where N_2-fixation was reported *i.e.* in slips treated with 2,4-D and *Azospirillum*, maximum activity was observed (Table 1). Elanchezhian and Panwar (1997) reported the presence of leghemoglobin in wheat *para*-nodules induced by 2,4-D and inoculated with *A. brasilense.* This is further supported by the reports on the existence of rice haemoglobins (Peter et al., 1997) and their function as O_2 carrier in metabolically active tissues (Anderesson et al., 1996).

Effect of 2,4-D and inoculation with *Azospirillum brasilense* on leaf chlorophyll content and nitrate reductase activity in the nodulated citronella

Total chlorophyll concentration was maximum in seedlings treated with both 2,4-D and *A. brasilense* at both 30 and 60 DAT (Table 2). Citronella slips treated with *Azospirillum* only also showed a higher chlorophyll concentration compared with the control and 2,4-D treated seedlings. The nitrate reductase activity was also higher in plants treated with *Azospirillum* either alone or

Table 2. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on leaf chlorophyll content and nitrate reductase activity in the nodulated citronella after transplantation in pots

Treatment	Chlorophyll content (mg/g F.W.)		Nitrate reductase activity (µ mol NO_2/g F.W./h)	
	30 DAT	60 DAT	30 DAT	60 DAT
Control	0.224	0.227	0.025	0.026
2,4-D	0.203*	0.196*	0.023*	0.019*
Azospirillum	0.385*	0.243*	0.028	0.031*
2,4-D+ *Azospirillum*	0.452*	0.767*	0.033*	0.050*

*Values were significant at 5% level of probability

Table 3. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on Glutamine synthetase (GS) and glutamate synthase (GOGAT) activities in the nodulated citronella after transplantation in pots

Treatment	Glutamine synthetase (µ mol /g F.W./h)		Glutamate synthase (µ mol NADH oxi/g F.W./min)	
	30 DAT	60 DAT	30 DAT	60 DAT
Control	61.45	69.78	65.09	71.86
2,4-D	58.71*	65.77*	58.01*	64.45*
Azospirillum	66.25*	73.41*	67.09*	79.81*
2,4-D+ *Azospirillum*	76.86*	84.56*	78.88*	98.90*

*Values were significant at 5% level of probability

Table 4. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on Photosynthetic rate and stomatal conductance activity in the nodulated citronella after transplantation in pots

Treatment	Photosynthetic rate (µ mol $CO_2/m^2/S$)		Stomatal conductance (m/S)	
	30 DAT	60 DAT	30 DAT	60 DAT
Control	4.70	4.40	0.06	0.41
2,4-D	3.76*	3.10*	0.03*	0.19*
Azospirillum	9.82*	11.08*	0.09*	0.51*
2,4-D+ *Azospirillum*	11.23*	12.32*	0.12*	0.75*

*Values were significant at 5% level of probability

in combination with both 2,4-D and *Azospirillum* than uninoculated control at both 30 and 60 DAT (Table 2). These results are in accordance with the earlier findings by Elanchezhian and Panwar (1997) in wheat plants in which *Azospirillum* enhanced the chlorophyll content and nitrate reductase activity.

Effect of 2,4-D and inoculation with *Azospirillum brasilense* on Glutamine synthetase (GS), glutamate synthase (GOGAT), photosynthetic rate and stomatal conductance activities in the nodulated citronella

Plants treated with *Azospirillum* along with 2,4-D (nodulated plants) showed increased activity of GS and GOGAT in leaves (Table 3), and increased photosynthetic rate and stomatal conductance when compared with uninoculated controls at different stages of growth (Table 4).

Effect of 2,4-D and inoculation with *Azospirillum brasilense* on oil yield

Data pertaining to the yield parameters (Table 5) showed that the inoculation with *A. brasilense* alone or along with 2,4-D contributed to the yield increase. Combination of 2,4-D and inoculation with *Azospirillum* produced higher

Table 5. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on oil yield after transplantation in pots

Treatment	Leaf length (cm)	Oil yield (%)
Control	112.33	0.96
2,4-D	97.30*	0.64*
Azospirillum	103.67*	1.35*
2,4-D+Azospirillum	110.33*	1.61*

*Values were significant at 5% level of probability

Table 6. Effect of 2,4-D and inoculation with *Azospirillum brasilense* on N content in leaf, stem and root of nodulated citronella after transplantation in pots

Treatments	Nitrogen content (%)		
	Stem	Leaf	Root
Control	37.21	24.65	82.05
2,4-D	33.08*	22.06*	56.27*
Azospirillum	62.05*	44.07*	70.05*
2,4-D+ *Azospirillum*	69.78*	44.45*	89.28*

*Values were significant at 5% level of probability

number of leaves per plant with increased leaf length, leaf weight and higher oil per leaf. Yield increases obtained in inoculated plants, however have been attributed to biological N_2-fixation and may also be due to the production of growth substance by the colonizing bacteria. Increased N supply through N_2-fixation, higher nitrate uptake (Subba Rao et al., 1985; Panwar, 1993) and higher chlorophyll content in the inoculated plants (Panwar and Elanchezhian, 1998) might have contributed to the increase in oil yield.

Effect of 2,4-D and inoculation with *Azospirillum brasilense* on N content in leaf, stem and root of nodulated citronella

The nitrogen content in the leaves was also increased with the inoculation of *Azospirillum* with greater N content being in the 2,4-D and *Azospirillum* inoculated plants (Table 6). The enhanced N content in leaves due to inoculation confirms the N_2-fixation and transfer of fixed N. The increased N content may be attributed to greater mineral uptake, nitrogenase activity and NR activity (Kennedy and Tchan, 1992; Ridge et al., 1992; Panwar, 1993).

The overall decrease in all the physiological parameters with only 2,4-D treated seedlings was due to poor root and shoot growth, root morphogenesis and nodulation without nitrogenase activity and leghemoglobin which resulted into reduced biomass production, oil yield and N uptake. The inoculation with N_2-fixing bacteria without any 2,4-D treatment showed no effect on root and shoot growth and nodulation but caused the induction of nitrogenase activity and leghemoglobin at lower rate which enhanced the yield compared to uninoculated treatments. However, inoculation with N_2-fixer in addition to 2,4-D treatment caused nodulation, induction of nitrogenase activity and leghemoglobin content that lead to the increased N_2-fixing which contributed to the higher chlorophyll content and NR activity leading to higher oil yield and N_2 content in leaves.

Based on the findings in the present study it may be concluded that the prospects for effective microbial biofertilizers for non-leguminous crops such as *C. winterianus* appears to be bright. *Azospirillum* inoculation benefits plant growth and also increases yield by improving root development (data not shown) and uptake. Also biological N_2-fixation by *Azospirillum* contributes to a significant amount of nitrogen to plants.

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