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Full Length Research Paper

Microscopic morphology of nitrogen fixing paranodules on wheat roots

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Symbiotic nitrogen fixation is an environmentally benign and inexpensive means of providing plants with nitrogen, but is currently not possible in non-legume grain crops. This study examines nitrogen fixing paranodules that developed on wheat (*Triticum aestivum*) roots after treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) or *Bacillus polymyxa* 43, a mixed culture of diazotroph bacteria *Xanthomonas* sp. and *Arthrobacter* sp. (XA) or their combination. The formation of paranodules 1 mm in diameter was observed two weeks after planting. The size of paranodules did not increase during subsequent plant growth. Light microscopic examination and scanning electron microscope showed a large number of bacteria within the mucigel. In ultra thin sections of the nodule, bacterial cells were found in the intercellular space of the paranodules. In the latter case, most microorganisms were oriented along the plant cell wall. The active functioning of introduced cultures within the paranodules was evidenced by the presence of dividing bacterial cells and bacteria surrounded by zones of lysis. The maximum nitrogen fixing activity was observed in wheat co-treated with 2,4-D and mixed culture of XA. This study supports the ability of a current wheat variety to form root paranodulation and nitrogen fixation by a naturally occurring soil diazotroph, and to benefit from the relation by increased biomass and protein.

Key words: *Arthrobacter* sp., biological nitrogen fixation, paranodule, wheat, *Xanthomonas* sp.

INTRODUCTION

One of the major factors limiting yield of agricultural crops is the ability of soil to supply nitrogen (N) in available forms for plant uptake. Observation of nitrogen fixation in the roots of grasses and non leguminous crops in the 1970s and observation of plant growth-promoting capabilities of *Azospirillum* initiated a wave of field experiments (Francisco and Akao, 1993). It is now well known that the use of different diazotrophic bacteria (*Agrobacterium radiobacter*, *Azosprillum lipoferum*, *Azosprillum brasilense*, *Azotobacter chroocaccum*, *Bacillus* sp., etc.), which were isolated from the rhizo-sphere of wheat plant can improve plant growth (Kennedy and Techan, 1992).

The creation of artificial nitrogen-fixing symbiotic association with plants that are not able to form them in a natural way is one of the most promising approaches to enhance the efficiency of the biological fixation of nitrogen in agriculture (Kennedy et al., 1997). There is evidence that some cereal plants are capable of forming endosymbioses with nitrogen-fixing bacteria through para-nodulation (Coking et al., 1990; Elanchezhian and Panwar 1997). In such endosymbioses, the role of microsymbiont may be played not only by rhizobia but also by bacteria of the genera *Azospirillum, Azotobacter, Derxia* and *Klebsiella* (Christisnsen and Vanderleyden, 1994; Elanchezhin et al., 1997). In recent years, several reports have appeared describing attempts to induce the formation of new growth referred to as paranodules on the roots of several non-legume plants including rice, wheat, barley and rape seed (Christisnsen and Vanderleyden, 1994; Glagoleva et al., 1996; Hardy et al.,

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **XA,** *Arthrobacter* sp.

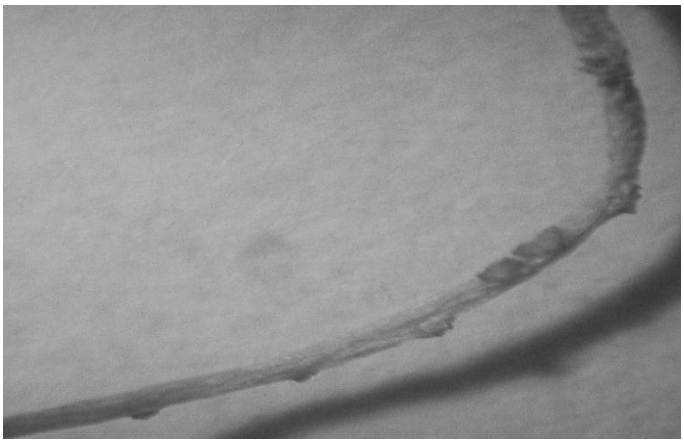


Figure 1. Fragment of a wheat root with p-nodules.

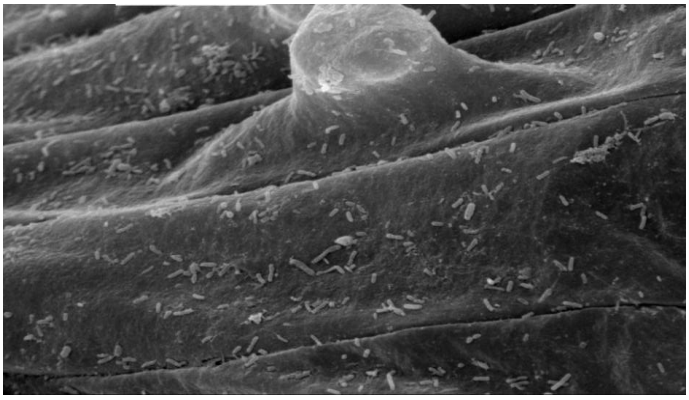


Figure 2. Colonization of wheat root surface by natural mixed bacterial culture (*Arthrobacter* sp. + *Xanthomonas* sp.) (×2000).

1968). Pereg-Gerk et al. (2000) found that the strains of *Azospirillum brasilense*, colonized both the crevices surrounding the sites of lateral root emergence in wheat and those crevices associated with the paranodules appearing after 2,4-dichlorophenoxyacetic acid (2,4-D)-treatment. However, the colonization of paranodules was more extensive than that of lateral root emergence sites in plants that were not treated with 2,4-D (Francisco and Akao, 1993).

The aim of this research was to induce formation and diazotroph colonization of paranodule on wheat roots, describe the ultrastructure of these interactions and determine nitrogen fixation activity in natural condition.

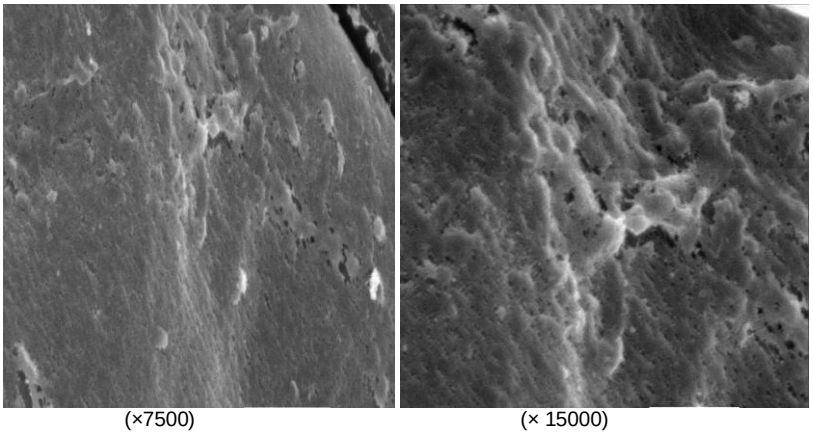
MATERIALS AND METHODS

Experiments were performed with winter wheat (*Triticum aestivum*) var. Pamiati phegina. Three soil-isolated organisms were used, one *Xanthomonas* sp. and one *Arthrobacter* sp. isolated from the rhizosphere of wheat, and one pectolytic bacteria *Bacillus polymyxa* 43 isolated from native range soil near Moscow Russia. Isolates were identified according to Bergey's Manual (Peter et al., 1984). Wheat plants were grown in an open-lattice shade, in 2 kg sandy clay loam soil at pH 6.5, topsoil from Chashinkovo experimental station of Moscow State University, Russia. The experiment was carried out using six treatments with seven replications: no treatment (CON), 0.1 2,4-D Micg.L^{-1} in potting soil and no bacterial inoculation (2,4-D), *B. polymyxa* 43 inoculation (BP), mixed culture inoculation of diazotrophic *Xanthomonas* sp. and *Arthrobacter* sp. (XA), mixed culture of XA plus 2,4-D (XA24D), and inoculation with both the diazotrophic bacteria (*Xanthomonas* sp. and *Arthrobacter* sp.) and pectolytic *B. polymyxa* 43 (XABP). At sowing and after emergence, pots received the following solutions twice according to their treatments (during sowing after emergence): 1) 134 (67 + 67) ml Fedorov–Kalininskaya (FK) (gram/liter K_2HPO_4 4.5, KH_2PO_4 3, $(NH_4)_2SO_4$ 2, Pectin, five amino acid, 1/5 3% $MgSO_4$ and agar 15); 2) 67 ml F.K. + 67 ml suspension 2,4-D; 3) 67 ml suspension of pectolitic bacteria (gram/liter: agar, 20 glucose; 10 K_2HPO_4 1.8, $MgSO_4$ *$7H_2O$ 0/3, $CaCl_2$*$6H_2O$ 0/01, NaCl 0/5, $FeCl_3$*$6H_2O$ 0/01) + 67 ml F.K; 4) 67 ml of the suspension mixed with nitrogen-fixing culture + 67 ml of F.K. medium; 5) 67 ml of XA culture + 67 ml of F.K. medium; 6) 67 ml of XA + 67 ml of the suspension of pectolitic bacteria. All mediums (suspension) were made sterile. Nitrogen-fixing activity was assayed by acetylene reduction assay (Hardy et al., 1968). Plants were cut at the soil line and air-dried before analysis. Total nitrogen and protein nitrogen contents of the aboveground plant matter were determined in triplicate by the Kjeldahl method. Results were statistically analyzed using a costat.

Observation on paranodules was carried out by using an Opton light microscope, a Jeol-100B transmission electron microscope, and an S-405A scanning electron microscope. The paranodule specimens for transmission electron microscopy were fixed with a 2% solution of glutaraldehyde in a cacodylate buffer, post fixed with a 2% solution of OsO_4 in the corresponding buffer for 4.5 h, dehydrated in a series of alcohol solutions of increasing concentration, and embedded in araldite epoxy resin. Thin sections were contrasted with lead citrate. For scanning electron microscopy, the preparations were fixed for 30 min in a 2% solution of glutaraldehyde in phosphate buffer, dehydrated in a series of alcohol solutions of increasing concentration, then in pure acetone, and dried in an HCP-2 Critical Point Dryer in an atmosphere of CO_2 at 35°C at a pressure of 100 atm. Then, material was coated with platinum-palladium alloy in argon atmosphere using an IB–3 Ion Coaster to a coating thickness of 150 Å.

RESULTS AND DISCUSSION

Wheat roots paranodules were formed after 2,4-D application or inoculation with a diazotrophic mixed culture (*Arthrobacter* sp. + Xanthomonas sp.). More paranodules were formed in XA 2,4-D treatment (Figure 1). Paranodules were formed two weeks after inoculation. On day 20, the paranodules were clearly noticeable and selected for cytological study. Scanning electron microscopy of roots showed surface colonization by bacteria (Figure 2) and a mucoid layer on the surface of the paranodules (Figure 3). Mucigel might create a micro environment for nitrogen-fixing bacteria and this may enhance the penetration of bacteria into plant tissues and colonization. This mucigel may also limit oxygen diffusion to the paranodules and bacteria, improving the potential activity of the oxygen-sensitive nitrogenase enzyme. One of the dominant bacteria forms was V-shaped cells, which is characteristic of coryneform bacteria (Figure 4). In ultra thin sections of the paranodules, bacterial cells were found both in the intercellular spaces (Figure 5). Most



(×7500) (× 15000)

Figure 3. Scanning electron micrographs of wheat root with mucigel.

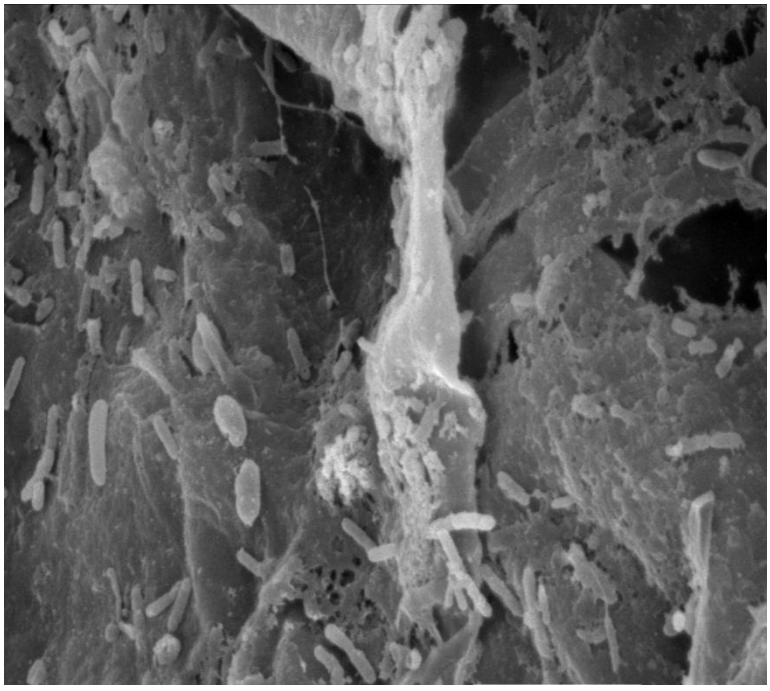


Figure 4. Scanning electron micrograph of wheat root (×5000).

bacteria cells were oriented along the plant cell wall (Figure 6). This could maximize the area of their contact with the plant cell and increase potential nutrient exchange and nitrogen fixation activity. Bacteria in the intercellular spaces were active and alive, indicated by the presence of dividing bacteria cells (Figure 7) and surrounded by zones of lysis (Figure 8). The obtained figures show that non leguminous plants can be induced to form paranodules, and that nitrogen fixing bacteria can colonize and reproduce within the induced paranodules.

The acetylene reduction assay of the roots showed that its nitrogen-fixing activity was maximum at 0.98 n mol C_2H_4 g^{-1} root h^{-1} on day 31 of plant growth in XA (Figure 9). Treatments CON and 2,4-D without addition of bacteria had no acetylene reduction activity. Wheat biomass was also greatest in XA (Figure 10). When plants were treated with the mixed nitrogen-fixing culture (*Xanthomonas* sp. + *Arthrobacter* sp.) plus 2,4-D or *B. polymixa* 43, nitrogen fixation and plant biomass were lower. This is contrary to the findings of Elanchezhian and Panwar (1997) who found acetylene reduction, wheat biomass and grain yield response in the order: 2,4-D plus *Azospirillum* > *Azospirillum* alone > 2,4-D or control. The results of the experiment show that nitrogen fixation activity of the bacteria on the wheat roots did not depend on pectolytic enzymes, BP but rather, the

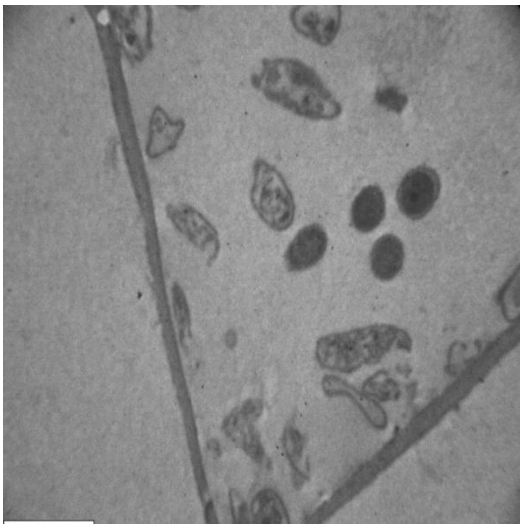


Figure 5. Electron micrograph of ultrathin section of wheat paranodules and bacteria in intercellular space (PCW, plant cell wall) (×10000).

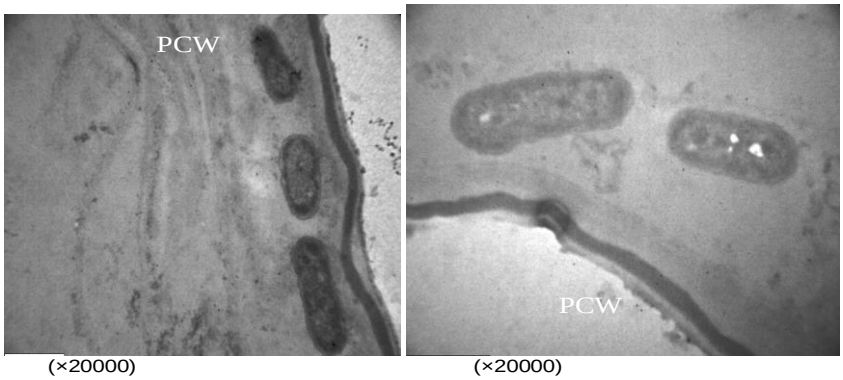


Figure 6. Electron micrograph of bacteria inside cells of wheat paranodules (PCW, plant cell wall).

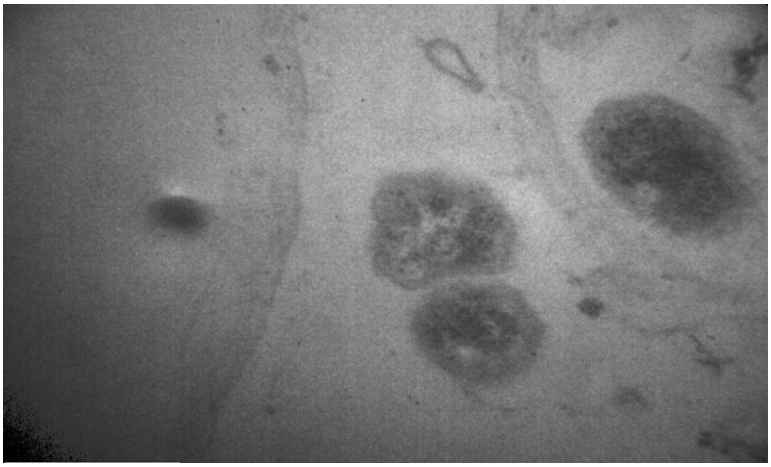


Figure 7. Electron micrograph of divided bacterial cells within wheat root (PCW, plant cell wall) (×20000).

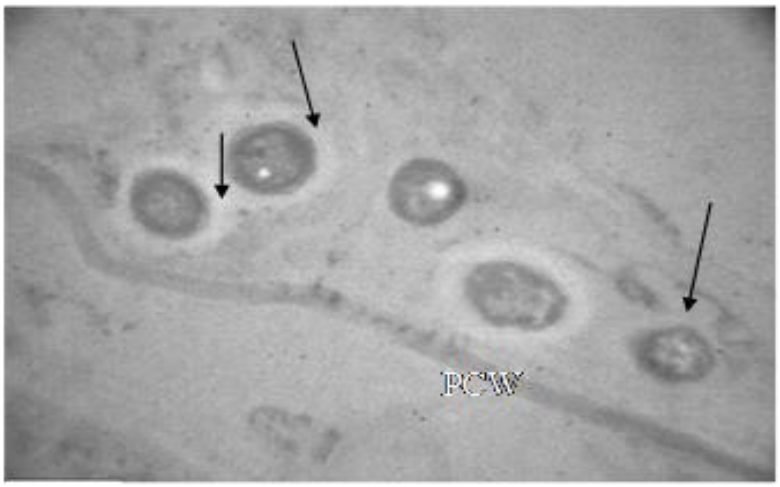


Figure 8. Electron micrograph of bacteria and lysis zone within wheat root during para nodulation (PCW, plant cell wall) (×20000).

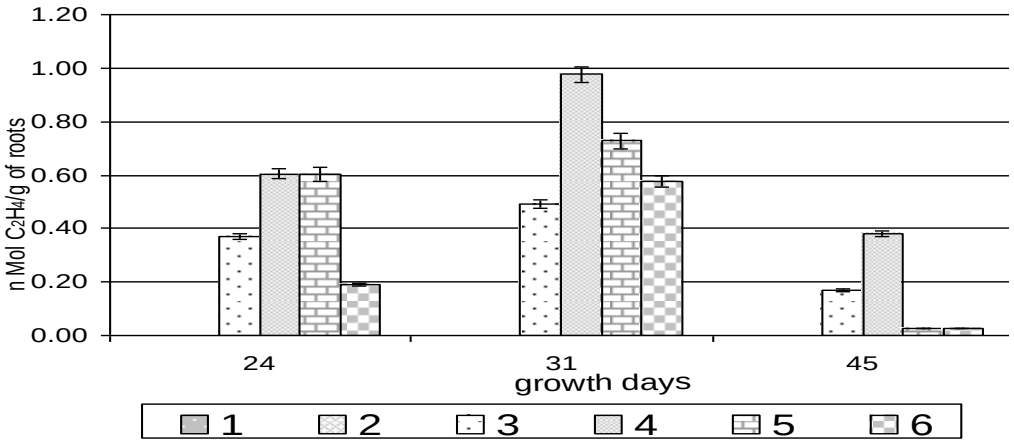


Figure 9. Dynamics of nitrogen-fixation activity on wheat roots in a model experiment.

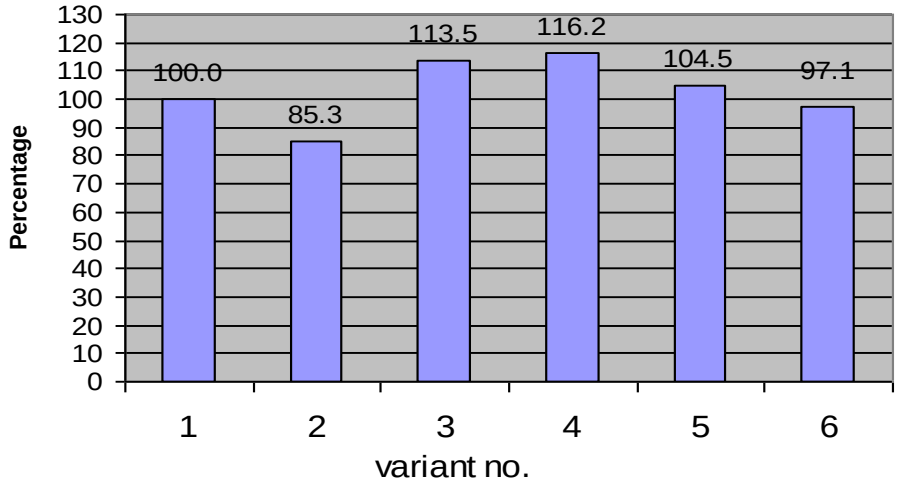


Figure 10. The effect of treatments on aboveground wheat biomass (as a percentage of the control).

Table 1. The effect of treatments on total plant nitrogen and protein nitrogen concentration (mean ± SE, n = 7).

Experimental variant	Experimental variant	Total nitrogen (%)	Protein nitrogen (%)	Protein (%)
1	Control	0.84 ± 0.06	0.58 ± 0.01	3.64 ± 0.06
2	2,4-D	0.88 ± 0.08	0.59 ± 0.05	3.68 ± 0.29
3	*Bacillus polymyxa 43*	0.82 ± 0.00	0.53 ± 0.10	3.31 ± 0.06
4	*Arthrobacter* sp. + *Xanthomonas* sp.	0.88 ± 0.03	0.63 ± 0.03	3.93 ± 0.19
5	*Xanthomonas* sp. + *Arthrobacter* sp. + 2,4-D	0.81 ±0.02	0.51± 0.04	3.17 ± 0.27
6	*Xanthomonas* sp. + *Arthrobacter* sp. + *Bacillus polymyxa* 43	0.90 ±0.05	0.61± 0.13	4.31 ± 0.79

2,4-D, 2,4-Dichlorophenoxyacetic acid.

addition of BP produced an adverse effect on the plant. Total nitrogen and protein nitrogen concentration of the above ground wheat biomass was not significantly affected by the treatments (Table 1).

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