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# Formation of artificial Nitrogen-fixing bacteria symbioses with wheat

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## Abstract

*Objective*: Many bacteria have positive effects on plant growth through mechanisms such as nitrogen fixation, production of phytohormones and suppression of pathogenic microorganisms. This study investigated the role of biological nitrogen fixation (BNF) in promoting growth of wheat through improved soil fertility.

*Methodology and results*: Wheat (*Triticum aestivum* var. pamiati phegina) plants grown in unsterile soil were inoculated with abiogenic nodulation agent (ANA) (2, 4 – dichlorophenoxyacetic acid or 2.4-D which acts as a herbicide when used at high rates, or a plant growth hormone when was used in low concentration); biogenic nodulation agent (BNA) (*Bacillus polymyxa* 43) and a mixed culture of nitrogen – fixing bacteria (*Xanthomonas sp*. + *Arthrobacter sp*.). The formation of p-nodule was observed two weeks after planting. Nodules formed measured about 1 mm in diameter. The size of p-nodules did not increase during subsequent plant growth. Inoculation of wheat with 2.4-D or bacteria had significant effects on dry weight and height of wheat plants and the associated nitrogen fixation activity. The maximum nitrogen - fixing activity was observed when wheat was inoculated with mixed cultures of diazotroph bacteria (*Xanthomonas sp. + Arthrobacter sp*.).

*Conclusion and application of findings*: This study achieved creation of artificial nitrogen-fixing symbiotic association between bacteria and wheat plants under laboratory conditions. Such associations could be exploited for formation biofertilizers that increase nutrient availability to plants.

*Key words:* Wheat, bacteria, inoculation, diazotroph, pectolitic, para-nodule

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## Introduction

Cereals, among them wheat (*Triticum aestivum)* are the world's major source of food for humans. Global food security depends on reaching and sustaining higher than current yield levels, which is not possible without use of substantial additional nutrient inputs, especially for wheat. Nitrogen fertilizer is important in wheat production since N is the main factor limiting productivity. Since agriculture is expected to move towards environmentally sustainable methods (Glagoleva et al., 1997), much attention has been paid to natural methods of meeting plants nitrogen requirements. One possibility is to increase the input of biological nitrogen.

The use of different diazotrophic bacteria, e.g. *Agrobacterium radiobacter, Azosprillum lipoferum, A.brasilense, Azotobacter chroocaccum* and *Bacillus azotofixans* isolated from the rhizosphere of wheat plants may enhance plant growth. Globally, biological N fixation as a part of the total nitrogen input to support crop growth has been on the decline. A significant reduction in the use of inorganic nitrogen fertilizers could be achieved if biological nitrogen fixation could be applied on cereal crops as it is applied on legume crops.

The most efficient biosystems for the fixation of molecular nitrogen is the symbiotic association of leguminous plants with bacteria of the genus *Rhizobium*; non-legumes (such as alder, sea buckthorn and raspberry) with actinomycetes of the genus Frankia, and fern Azolla with cyanobacteria *Anabaena azolla.* In these associations, nitrogen-fixing bacteria grow in specific structures providing beneficial conditions for diazotrophs.

The creation of artificial nitrogen-fixing symbiotic associations 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 biological fixation of nitrogen in agriculture. The aim of the present work was to induce artificial symbiosis in wheat plants under laboratory conditions.

## Materials and methods

Experiments were performed with winter wheat (*Triticum aestivum)* var. Pamiati phegina. For inoculation of seedlings a nitrogen-fixing strain of mixed culture *Xanthomonas sp.* + *Arthrobacter sp.* isolated from the rhizosphere of wheat and pectolytic bacteria from the rhizosphere were used. These cultures play the role of biogenic agents. Identification was performed according to Bergy's Manual (Peter et al., 1984). Wheat plants were grown in vases containing 2 Kg sandy-clay-loam soil at pH 6-7 sampled from the arable layer at the experimental plot of the Chashinkovo Biostation of Moscow State University (Moscow, Russia). Other than the bacterial treatments plants were also treated with 2, 4D, a plant growth stimulant at a concentration of 0.1 µg/l as an "abiogenic agent".

The experiment was carried out in 6 treatments, each with 7 replications. Treatments were (1) plants without any treatment (control); (2) plants without bacterial inoculation but with 2.4.D; (3) plants inoculated with pectolytic bacteria (*Bacillus polymyxa* 43); (4) plants inoculated with mixed culture of diazotrophic bacteria (*Xanthomonas sp.* + *Arthrobacter sp*); (5) plants inoculated with mixed cultures of bacteria (*Xanthomonas sp.* + *Arthrobacter sp.)* + 2.4.D; (6) plants inoculated with mixed culture of diazotrophic bacteria (*Xanthomonas sp.* +*Arthrobacter sp*). + *Bacillus polymyxa* 43.

During sowing and after germination, the soil contained in the seedling pans was supplemented with correspondingly; (1) 134 (67 +67) ml of Fedrov-Kalinskaya (F.K.) liquid medium consisting of 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 gram per litre (g/l ); (2) 67 ml of F.K. medium; (3) 67 ml suspension of pectolytic bacteria + 67 ml of F.K. medium; (4) 67 ml of the suspension mixture of nitrogen-fixing culture + 67 ml of F.K. medium; (5) 67 ml of the suspension mixture of nitrogen-fixing culture + 67 ml of the suspension of pectolitic bacteria.

All of the media (suspensions) were sterile. The nitrogen-fixing activity was assayed by acetylene reduction method. For this purpose plants were extracted from the pans and the roots separated from the shoot and placed into non –sterile 5 ml flasks (3-4 plants per flask). After injection of 1 ml of acetylene into the flasks and 60 minute incubation at 28 °C under illumination, the ethylene content was measured by the acetylene reduction method using a chrom-41 (Czechoslovakia) gas chromatograph with flame- ionization detector. The results were statistically analyzed using Microsoft EXCEL and CASTAT. Treatment means with significant differences were separated by the Least Significant Difference (LSD) test.

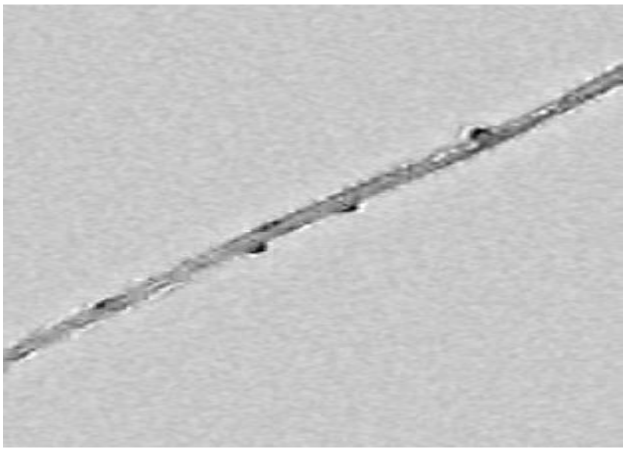

Figure 1: Formation of para-nodules on roots of wheat plants.

## Results and discussion

The formation of p-nodule was observed 2 weeks after planting when the nodules measured about 1 mm in diameter. The size of p-nodules did not increase further during subsequent plant growth. Para nodules formed in all treatments except the control plants. The type of treatment had no significant influence on the number, morphology and the formation time of Para nodules. It is know that formation of p-nodules on the roots of non symbiotrophic plants can also occur without involvement of microorganisms, solely under the influence of an auxin-like inducer of para-nodulation.

Table 1: Mean weight (g) of wheat plants treated to stimulate nodulation for nitrogen fixation..

| Treatment | Days after planting | | | |
|---|---|---|---|---|
| | 22 | 29 | 36 | 43 |
| 1 | 0.0494 b | 0.0839 b | 0.1089 bc | 0.1506 b |
| 2 | 0.0632 a | 0.13685 a | 0.159076 a | 0.19757 a |
| 3 | 0.0552 ab | 0.0851 b | 0.10948 bc | 0.20838 a |
| 4 | 0.0575 ab | 0.0874 b | 0.094001 c | 0.114137 d |
| 5 | 0.06095 a | 0.07935 b | 0.12167 b | 0.14137 bc |
| 6 | 0.0355 c | 0.0759 b | 0.091025 c | 0.12178 cd |
| P | * | * | * | * |

*The data are an average of 5 plants. Treatments were (1) plants without any treatment (control); (2) plants without bacterial inoculation but with 2.4.D; (3) plants inoculated with pectolitic bacteria (*Bacillus polymyxa* 43); (4) plants inoculated with mixed culture of diazotrophic bacteria (*Xanthomonas sp.* + *Arthrobacter sp*); (5) plants inoculated with mixed cultures of bacteria (*Xanthomonas sp.* + *Arthrobacter sp.)* + 2.4.D; (6) plants inoculated with mixed culture of diazotrophic bacteria (*Xanthomonas sp.* +*Arthrobacter sp*). + *Bacillus polymyxa* 43. Different letters after treatment means along the columns indicate significant differences as determined by the Least Significant Difference (LSD) test. Overall significant between treatments indicated by $p \leq 0.05$ = *

After further examination by scanning electron microscopy, the formed p-nodules were found on the main root and around the lateral root (Fig. 1). These typical grayscale scanning electron micrographs depicting various morphological features of nodules were clear on wheat plants that were treated with mixed cultures of *Xanthomonas sp.* and *Arthrobacter sp.* Inoculation of wheat seeds and wheat seedling had significant effects on plant dry weight (table 1) and height (table 2). The maximum weight and height were observed when plants were inoculated either with 2, 4D or *Bacillus polymyxa* 43. This response is probably because these treatments acted as auxin and stimulated plant growth when assessed at 36 and 43 days after sowing.

The use of 2, 4D together with mixed culture (treatment 5) increased dry weight more than when only the mixed culture (treatment 4) was used. Inclusion of 2, 4D also led to an increase in the nitrogenous activity in the wheat rhizosphere (fig.2).

The nitrogen fixing activity of roots of wheat plants grown in non- sterile soil that was treated with mixed culture + 2, 4D was at a maximum on the 43rd day of plant growth (fig.2). This observation is important in that it demonstrates that with appropriate treatment it is possible to form nodules on wheat. The results further showed that nitrogen fixation activity in plants with treatment 5 (2,4D + mixed culture) was twice as high as that of treatment 4 (mixed culture only) that shows that 2, 4D stimulates nitrogenous activity. The agent 2, 4D, acting as an auxin, could stimulate bacterial colonization of the nodular structures modified from the lateral roots of the wheat plants.

Table 2: Mean height of wheat plants (cm) treated to stimulate nodulation for nitrogen fixation.

| Treatment | Days after planting | | | |
|---|---|---|---|---|
| | 22 | 29 | 36 | 43 |
| 1 | 16.83 bc | 19.85 b | 21.43 bc | 26.7 b |
| 2 | 17.93 bc | 29.05 a | 30.1 a | 33.7 a |
| 3 | 18.1 bc | 20.35 b | 24.3 b | 35.07 a |
| 4 | 20 a | 21.65 b | 21.66 c | 22.35 c |
| 5 | 19.77 ab | 20.7 b | 21.05 b | 22 c |
| 6 | 15.6 c | 19.1 b | 20.4 c | 25.46 b |

Different letters after means along the columns indicate significant differences at $p \leq 0.05$, LSD.

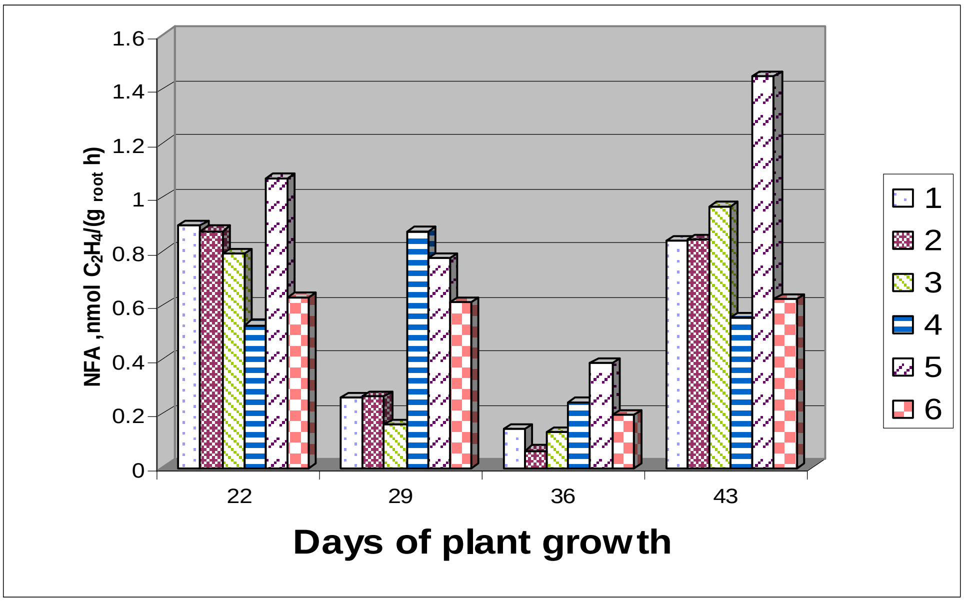


Figure 2: Dynamics of the nitrogen-fixing activity (NFA) of wheat roots treated to stimulate nodulation.

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