Effect of salinity tolerant PDH45 transgenic rice on physicochemical properties, enzymatic activities and microbial communities of rhizosphere soils

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The effect of genetically modified (GM) plants on environment is now major concern worldwide. The plant roots of rhizosphere soil interact with variety of bacteria which could be influenced by the transgene in GM plants. The antibiotic resistance genes in GM plants may be transferred to soil microbes. In this study we have examined the effect of overexpression of salinity tolerant pea DNA helicase 45 (PDH45) gene on microbes and enzymatic activities in the rhizosphere soil of transgenic rice IR64 in presence and absence of salt stress in two different rhizospheric soils (New Delhi and Odisha, India). The diversity of the microbial community and soil enzymes viz., dehydrogenase, alkaline phosphatase, urease and nitrate reductase was assessed. The results revealed that there was no significant effect of transgene expression on rhizosphere soil of the rice plants. The isolated bacteria were phenotyped both in absence and presence of salt and no significant changes were found in their phenotypic characters as well as in their population. Overall, the overexpression of PDH45 in rice did not cause detectable changes in the microbial population, soil enzymatic activities and functional diversity of the rhizosphere soil microbial community.

Introduction

The soil salinity has become a major problem affecting plant productivity worldwide. Moreover, a rise in sea levels due to global warming is likely to intensify these problems. Therefore there is no alternative but to produce more saline-tolerant plant varieties, particularly for rice, the staple crop (Oryza sativa L.). Since salinity tolerance is controlled by multiple genes, the overexpression of a single gene resulting in field-level tolerance in rice is limited.1 On the other hand, the overexpression of the salt-inducible pea DNA helicase, PDH45, in tobacco and rice (IR64) resulted in strong NaCl stress tolerant phenotype even when irrigated continuously with 200 mM NaCl, without affecting the yield.2,3 PDH45 is a unique member of this family as it contains DESD and SRT instead of the typical DEAD/H and SAT in motifs V and VI, respectively.4

A specific microbial community is found in the zone around the root (called rhizosphere) that is influenced by the plant. The presence of antibiotic resistance genes in genetically modified (GM) plants as selection markers have raised questions about the possible transfer of these genes to indigenous microbes in the soil. For this reason, environmental risk assessment of GM plants has been mainly focused on possible horizontal gene transfer (HGT) to relative plants or to the soil- and plant-associated microbial communities.5 It has been suggested that the rhizospheres could be altered in response to plant genetic transformation through HGT from GM plants to the indigenous soil microbes.6 The comparative studies assessing differences between the microbial communities living in the rhizospheres of GM and non-GM plants represent a first step in determining if the presence of transgenic material can produce changes in the environment. It is evident that when GM plants were grown in a place for a long time, they change the rhizospheric microbial metabolism, enzymatic status causing negative effects on soil quality, structure and function and affecting the enzyme synthesis in microbes and activity as well as soil processes such as litter decomposition and mineralization.7 There were some transient differences in the rhizospheric microbial community between soils cultivated with transgenic and non-transgenic plants. It has been shown that the Cry1Ab (gene from Bacillus thuringiensis subsp Kurstaki) protein is released in root exudates and binds rapidly onto surface-active particles in soil and become less accessible to microbial degradation but retains the insecticidal activity.8 Therefore, accumulation of these proteins in soil may influence soil biological processes and microbial community composition. Blackwood and Buyer (2004)9 used phospholipid fatty acid (PLFA) profiles
and community-level physiological profiles of microbes to determine whether growing Bt corn had any effect on soil microbial communities as compared with the growth of non-Bt corn. They found that the profiles of the microbial communities were heavily affected by soil type, but the effect of expression of the Bt gene in corn was small.

An analysis of the soil enzymatic activity is one of microbiological indicators of soil quality. Enzymes participate in numerous biochemical processes occurring in the soil and they are sensitive to all environmental changes caused by natural and anthropogenic factors.10 Enzymes are secreted by floral and faunal organisms, but most often they are produced by microorganisms in soil. The soil analysis therefore, includes the determination of the activity of intracellular enzymes, enzymes found on the cell surface and free enzymes. Their activity is related to the physical properties of the soil, organic matter content and the mechanism of action. The measurement of enzymatic activity in combination with the count of key microorganisms provides sensitive information about the changes occurring in the soil.11 Some well-developed techniques, such as traditional plate based, most probable number (MPN) and direct microscopic counts, as well as molecular-based procedures and fatty acid analyses, can be used for characterizing soil microorganisms and to evaluate possible influence of transgenic plant on soil ecosystem.12 The alteration of biological properties of the soil have often been proposed as early and sensitive indicators of soil ecological stress or other environmental changes.13 The assessment of microbial populations in combination with their activity provides more sensitive information than either activity or population analysis alone. The soil microorganisms like bacteria and fungi are the dominant organisms in soil and play central roles in the breakdown of organic matter, mineralization and fixation of nutrient, control of plant disease and amelioration of soil structure.14 The impacts on soil microbial communities are therefore an important aspect of environmental risk assessment especially to monitor transgenic insect-resistant plants.15

In the present study, we used overexpressing PDH45 T1 transgenic rice plants as our experimental system and we evaluated the changes in the rhizospheric soil of two different locations of India (New Delhi and Odisha, India) and soil microbial communities colonizing the rhizosphere of PDH45 transgenic rice plant in comparison to its non transgenic counterpart in the presence and absence of salt. In addition, the effects of PDH45-transgene on rhizospheric soil dehydrogenase activity (DHA), phosphatase, urease and nitrate reductase activities were also evaluated.

Results

Analysis of PDH45 T1 transgenic plants. The PDH45 transgenic plants has been analyzed and described earlier.2 Briefly, the PDH45 overexpressing transgensics rice plants (T1) performed well in water irrigated as well as in salt stress (200 mM NaCl) conditions. All the plants set viable seeds in both the conditions without yield loss (Fig. 1B). Furthermore, the T1 transgenic plants showed significantly higher levels of leaf chlorophyll, nutrient content, net photosynthetic rate and phenotypic expression and yield when irrigated with 200 mM NaCl as compared with the WT plants.2

Physico-chemical properties of the experimental soil. The physico-chemical properties of the rhizospheric soil of New Delhi, India and Odisha, India used in pots of WT as well as transgenic plants were lateritic were (Table 1). The soil type of New Delhi was sandy clay loam having sand 62%, slit 21%, clay 17%, bulk density of 1.43 gcm³, particle density of 2.67 gcm³. The soil pH was 8.2 and 9.1 in absence and presence of salt, respectively. The soil of Odisha was alluvial sandy loam having sand 71%, slit 18%, clay 11%, bulk density of 1.75 gcm³, particle density of 2.97 gcm³. The pH of the soil in absence and presence of salt was 6.53 and 7.1, respectively. The soil analysis of two soils showed organic carbon content of 0.45% and 0.42% available nitrogen 247 and 212 kg/ha, available phosphorus 48 and 22.02 kg/ha, available potassium 265, 102.06 kg/ha, available calcium 253 and 128.9 kg/ha, available magnesium 198 and 208 kg/ha and available sulfur 18.6 and 12.3 kg/ha in absence as well as presence of salt. The available sodium content was 6.53 and 7.1, respectively. The soil type of New Delhi was sandy clay loam having sand 62%, slit 21%, clay 17%, bulk density of 1.43 gcm³, particle density of 2.67 gcm³. The soil pH was 8.2 and 9.1 in absence and presence of salt, respectively. The soil of Odisha was alluvial sandy loam having sand 71%, slit 18%, clay 11%, bulk density of 1.75 gcm³, particle density of 2.97 gcm³. The pH of the soil in absence and presence of salt was 6.53 and 7.1, respectively. The soil analysis of two soils showed organic carbon content of 0.45% and 0.42% available nitrogen 247 and 212 kg/ha, available phosphorus 48 and 22.02 kg/ha, available potassium 265, 102.06 kg/ha, available calcium 253 and 128.9 kg/ha, available magnesium 198 and 208 kg/ha and available sulfur 18.6 and 12.3 kg/ha in absence as well as presence of salt. The available sodium content was 20 and 18 Kg/ha in absence of salt and 27 and 25 Kg/ha in presence of salt, both in case of the WT and the PDH45 transgenic rice rhizosphere.

Population of bacteria and nematodes in rhizosphere soil of WT and PDH45 transgenic T1 plants. Five types of different bacterial colonies were isolated from the rhizospheric soil (New Delhi and Odisha, India) and soil microbial communities...
found 51,55 and 49,53 × 10^5 cfu/g in WT and 48,52 and 48,50 colonies. The population dynamics of rhizospheric bacteria were summarized the morphological feature assessed for the isolated colonies. Size (ranged from 0.60–1.00 mm) and texture (gummy, not mucoid) (Table 2). Seven different types of bacterial colonies were isolated from the rhizospheric soil (Odisha) of WT control and WT salt pots. Six different types of bacterial colonies were isolated from rhizospheric soil of PDH45 transgenic control and PDH45 transgenic salt treated plants (Table 3). Table 2 and 3 summarizes the morphological feature assessed for the isolated colonies. The population dynamics of rhizospheric bacteria were found 51, 55 and 49, 53 × 10^5 cfu/g in WT and 48, 52 and 48, 50 × 10^5 cfu/g in PDH45 transgenic plants in absence and presence of salt respectively, (Table 4). The population of nematodes was 911.3, 845 and 917.4, 853 in each pot (app. 8 kg soil) in case of WT and 915.2, 842 and 920.3, 847 in PDH45 transgenic in the absence and presence of salt, respectively, (Table 4).

DHA, alkaline phosphatase, urease and nitrate reductase activity in rhizosphere soil. The effect of transgenic rice on rhizospheric soil enzymes, DHA, alkaline phosphatase, urease and nitrate reductase, enzyme assay were performed. On the basis of above enzymatic assay it was found that PDH 45 transgenic rice plant did not show a significant difference when compared with its WT counterpart both in the presence and absence of salt. In the absence of salt, PDH45 T1 transgenic and WT rice showed DHA activity values of 20.2 ± 1.5 and 20.1 ± 1.5 μg pNP g−1 soil h−1 in New Delhi and 16.5 ± 1.4 and 17.0 ± 1.6 μg pNP g−1 soil h−1 in Odisha, soil alkaline phosphatase activity were 170.4 ± 10.5 and 170.4 ± 10.5 μg pNP g−1 soil h−1 in New Delhi and 160 ± 11.4 and 163 ± 11.5 μg pNP g−1 soil h−1 in Odisha soil, urease activity as 154.7 ± 10.2 and 154.2 ± 10.2 μg urea hydrolysed g−1 h−1 in New Delhi soil and 140 ± 11.1 and 142 ± 11.3 μg urea hydrolysed g−1 h−1 in Odisha soil. The nitrate reductase activity were 0.5 ± 0.01 and 0.5 ± 0.01 μg NO2− Ng−1 h−1 in New Delhi soil and 0.7 ± 0.01 and 0.7 ± 0.01 μg NO2− Ng−1 h−1 in Odisha soil, respectively (Fig. 2 and 3). A slight difference in the enzymatic activities of salt treated soil were observed. In the presence of salt, values of DHA were 21.1 ± 1.5 and 21.1 ± 1.5 μg pNP g−1 soil h−1 in New Delhi and 16 ± 1.4 and 16.5 ± 1.4 μg pNP g−1 soil h−1 in Odisha soil. Alkaline phosphatase level was 165.1 ± 10.5 and 166.9 ± 10.5 mg pNP g−1 soil h−1 in New Delhi and 163 ± 10.4 and 160 ± 10.4 mg pNP g−1 soil h−1 in Odisha soil, urease activity as 154.2 ± 10.2 and 154.7 ± 10.2 μg urea hydrolysed g−1 h−1 in New Delhi soil and 140 ± 10.4 μg urea hydrolysed g−1 h−1 in Odisha soil. The nitrate reductase activity as 0.5 ± 0.01 and 0.5 ± 0.01 μg NO2− Ng−1 h−1 in WT and PDH45 transgenic rice plants, respectively (Figs. 2 and 3A–D).

**Discussion**

In the present study, we tried to analyze the effect of salt stress tolerant PDH45 transgenic plants on the rhizosphere soil to assess its potential as a candidate for field trials. Our results on PDH45 transgenic rice showed that introduction of transgenic plants did not alter the physico-chemical properties of rhizosphere soil, enzymatic activity and also the population of soil microflora.

The literature survey indicates variable effect of transgenic plants on rhizosphere soil. While a few reports have indicated
the adverse effects of the transgenic plants, few others have demonstrated no significant difference in composition of the rhizosphere. It has been reported that Bt corn affects the microbial communities, activities of some enzymes and microbe-mediated processes and functions in the soil. The study by Aira et al., (2010) on the microbial communities in maize rhizosphere states that the plant genotype (sul1 and sb2 genes) strongly influence the structure and growth of rhizosphere microbial communities. However, the results presented in this study are consistent with the experiment conducted with Bt rice. They did not find any significant differences in dehydrogenase and phosphatase activities, respiration, methanogenesis and in composition of fungal community in rhizosphere soil of transgenic and wild type rice. Oliveira et al., (2008) found that the microbial populations of the soil did not affect in presence of Bt maize. Likewise, some studies have proven that the long-term cultivation of transgenic plants did not affect the soil microbial diversity. It is interesting to note that in the present study, no significant differences were observed in enzyme activity in soil rhizosphere of PDH45 transgenic and non transgenic (WT) plants of same variety, which also correlates with the experiment on Bt cotton where except for dehydrogenase enzyme, the differences in the activity of alkaline phosphatase, nitrate reductase and urease enzymes between Bt and non Bt plants rhizosphere were statistically non-significant. No adverse effect was observed in MCM6 transgenic tobacco cultivation on soil enzymatic activities and rhizosphere microbial communities. From the above data, it is clear that the cultivation of transgenic plants in all cases did not affect on soil microbial community and enzymatic activity. It depends on the particular plants, techniques, protein and environmental conditions.

In this study we have tested the impact of PDH45 transgenic rice plant in two different soil and no significant variations were observed in population of bacteria and nematodes in PDH45 transgenic and non-transgenic rice rhizosphere. The observation of this study correlates to the observation reported earlier by Saxena and Stotzky (2001). They observed that transgenic corn crops had no apparent effect on nematodes.

Table 2. Colony characteristics of different isolates of rhizospheric soil (New Delhi, India) of pots

| Rhizospheric Soil (New Delhi, India) | Isolate no. | Form | Color | Elevation | Margin | Size(mm) | Consistency | Motility | Gram staining |
|-------------------------------------|-------------|------|-------|-----------|--------|----------|------------|---------|--------------|
| WT control                          | 1           | Circular | Brown | Low convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 2           | Circular | Brown | Convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 3           | Circular | Brown | Convex | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                     | 4           | Circular | Fluorescent | Flat | Entire | 0.65–0.75 | Not gummy | + | −ve |
|                                     | 5           | Circular | Off white | Flat | Entire | 0.60–0.75 | Mucoid | + | −ve |
| WT+ salt                            | 1           | Circular | Brown | Low convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 2           | Circular | Brown | Convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 3           | Circular | Brown | Convex | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                     | 4           | Circular | Fluorescent | Flat | Entire | 0.65–0.75 | Not gummy | + | −ve |
|                                     | 5           | Circular | Off white | Flat | Entire | 0.60–0.75 | Mucoid | + | −ve |
| PDH45 control                       | 1           | Circular | Brown | Convex | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                     | 2           | Circular | Brown | Low convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 3           | Circular | Fluorescent | Flat | Entire | 0.65–0.75 | Not gummy | + | −ve |
|                                     | 4           | Circular | Off white | Flat | Entire | 0.60–0.75 | Mucoid | + | −ve |
|                                     | 5           | Circular | White | Plicate | Entire | 0.95–1.00 | Gummy | + | −ve |
| PDH45+salt                          | 1           | Circular | Brown | Low convex | Entire | 0.60–0.75 | Gummy | + | −ve |
|                                     | 2           | Circular | Brown | Convex | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                     | 3           | Circular | Fluorescent | Flat | Entire | 0.65–0.75 | Not gummy | + | −ve |
|                                     | 4           | Circular | Off white | Flat | Entire | 0.60–0.75 | Mucoid | + | −ve |
|                                     | 5           | Circular | White | Plicate | Entire | 0.95–1.00 | Gummy | + | −ve |
|                                     | 6           | Circular | Brown | Low convex | Entire | 0.60–0.75 | Gummy | + | −ve |

Material and Methods

Plasmid construct and Agrobacterium-mediated transformation of rice. PCR amplified PDH45 ORF (1.2 Kb; amplified using Forward primer: 5’-GAG CTC ATG GCG ACA ACT TCT GTG G-3’ Reverse primer: 5’-GAG CTC GAG TTA TAT AAG ATC ACC AAT ATT C-3’) was cloned in pRT100 using XbaI restriction enzymes. The PDH45 plant expression cassette from pRT100 was then mobilized to binary vector pCAMBIA1301 using restriction enzyme PstI (Fig. 1A). Competent strain of Agrobacterium tumefaciens (LBA4404) was transformed with pCAMBIA1301-PDH45 construct as described earlier.
derived calli of rice IR64 were used for Agrobacterium-mediated transformation as described.21 Putative T₁ transgenic plants were transferred to pots containing vermiculite and were incubated in green house operating at 28°C, 16 h light at 100–125 μmolm⁻²s⁻¹ and 70–75% relative humidity. The plants were regularly irrigated with nutrient medium and were grown to maturity. The transgenic plants were then screened by PCR. The seeds from T₁ transgenic and wild type (WT) rice plants were gated with nutrient medium and were grown to maturity. The tolerance of plants to salinity was tested after 21 d in the presence and absence of salt (200 mM NaCl). Rhizosphere soil samples collected from each pot was transferred to plastic bags and plant debris was removed manually. Soil samples were then powdered and sieved and kept at 4°C, in dark for further analysis.

**Experimental site and soil sampling.** The pot experiments were conducted in the green house of International Centre for genetic Engineering and Biotechnology, New Delhi using two type of soils (New Delhi, India and Odisha, India). In triplicates, the T₁ transgenic and wild type (WT) rice plants were grown in earthen pots at 28°C, 16 h light at 100–125 μmolm⁻²s⁻¹ and 70–75% relative humidity. The plants were grown for 90 d to maturity and seeds were collected. The tolerance of plants to salinity was tested after 21 d in the presence and absence of salt (200 mM NaCl). Rhizosphere soil samples collected from each

| Rhizospheric Soil (Odisha, India) | Isolate no. | Form | Color | Elevation | Margin | Size (mm) | Consistency | Motility | Gram staining |
|----------------------------------|-------------|------|-------|-----------|--------|-----------|-------------|----------|--------------|
| WT control                       | 1           | Circular | White | Entire | 0.95–1.00 | Gummy | + | −ve |
|                                 | 2           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 3           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 4           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 5           | Circular | Off white | Flat | 0.60–0.75 | Mucoid | + | −ve |
|                                 | 6           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 7           | Circular | White | Entire | 0.90–1.00 | Gummy | + | −ve |
| WT+ salt                         | 1           | Circular | White | Entire | 0.95–1.00 | Gummy | + | −ve |
|                                 | 2           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 3           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 4           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 5           | Circular | Off white | Flat | 0.60–0.75 | Mucoid | + | −ve |
|                                 | 6           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 7           | Circular | White | Entire | 0.90–1.00 | Gummy | + | −ve |
| PDH45 control                    | 1           | Circular | Off white | Flat | 0.60–0.75 | Mucoid | + | −ve |
|                                 | 2           | Circular | White | Entire | 0.95–1.00 | Gummy | + | −ve |
|                                 | 3           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 4           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 5           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 6           | Circular | Brown | Entire | 0.60–0.75 | Gummy | + | −ve |
| PDH45+salt                       | 1           | Circular | Off white | Flat | 0.60–0.75 | Mucoid | + | −ve |
|                                 | 2           | Circular | White | Entire | 0.95–1.00 | Gummy | + | −ve |
|                                 | 3           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 4           | Circular | Brown | Entire | 0.85–0.90 | Gummy | + | −ve |
|                                 | 5           | Circular | Fluorescent | Flat | 0.65–0.75 | Not gummy | + | −ve |
|                                 | 6           | Circular | Brown | Entire | 0.60–0.75 | Gummy | + | −ve |

Measurement of soil pH and electrical conductivity of soil. To determine Eh and pH of the soil samples, 50 g of soil was suspended in 100 ml distilled, deionised water and stirred for 1 h at 100 rpm on a rotary shaker. The supernatant of the sample was then collected by centrifuged it at 10,000 g for 5 min. Eh (mS/cm) was recorded through a conductivity meter (Systronics) against 0.01 N KCl and pH was measured through a pH meter (Systronics).24
The available phosphorus content of the soil was determined as explained by Page et al., (1982). Soil (2.5 g) was taken in a 100 ml conical flask, a pinch of Olsen's reagent (0.5 M NaHCO₃) was added to it and the flask was shaken to thoroughly mix the ingredients. Five ml of filtered solution was taken in a 25 ml volumetric flask and 5 ml ammonium molybdate (0.5 N), 1 ml freshly prepared SnCl₂ (10 mM) solutions were added and the volume was made up to 25 ml by addition of distilled water. The optical density (OD) was measured at 660 nm.

The available potassium content of the soil was determined by method described by Jackson (1973). Soil (5 g) was taken in a 250 ml conical flask and 25 ml of 1 N CH₃COONH₄ solution was added to it. The flask was shaken for 30 min, the solution was filtered and the filtrate was diluted to 50 ml with 1 N CH₃COONH₄ solution and the data were measured by flame photometer (Bellstone) and the concentration was calculated by plotting the readings against the standard curve.

Sodium (Na) can be extracted with ammonium acetate solution in the same way as K, subsequently; Na in the extract can be determined by flame photometry. Certain elements, including Na, have the property that, when their salts are introduced into a flame, they emit light with a wavelength (color) specific to the element and of intensity proportional to the concentration.

The available sulfur content of the soil was determined by method described by Chesin and Yien (1951). Twenty grams of soil sample was taken in a 250 ml conical flask and 25 ml of 1 N CH₃COONH₄ solution was added to it. The flask was shaken for 30 min, the solution was filtered and the filtrate was diluted to 50 ml with 1 N CH₃COONH₄ solution and the data were measured by flame photometer (Bellstone) and the concentration was calculated by plotting the readings against the standard curve.

**Table 4. Population of nematodes and bacteria in rhizospheric soil of different pots**

| Rhizospheric soil (New Delhi, India) | Nematodes numbers/pots | Population of bacteria in pots (x 10⁶ cfu/g soil) | Rhizospheric soil (Odisha, India) | Nematodes numbers/pots | Population of bacteria in pots (x 10⁶ cfu/g soil) |
|-------------------------------------|------------------------|-----------------------------------------------|-----------------------------------|------------------------|-----------------------------------------------|
| WT control                          | 911.3 ± 1.7ᵃ            | 51 ± 0.14ᵃ                                    | WT control                        | 845 ± 1.9ᵃ            | 55 ± 0.16ᵃ                                    |
| WT+salt                            | 917.4 ± 1.9ᵃ            | 49 ± 0.12ᵃ                                    | WT+salt                           | 853 ± 1.7ᵃ            | 53 ± 0.15ᵃ                                    |
| PDH45 control                       | 915.2 ± 1.4ᵃ            | 48 ± 0.12ᵃ                                    | PDH45 control                      | 842 ± 1.7ᵃ            | 52 ± 0.14ᵃ                                    |
| PDH45+salt                         | 920.3 ± 1.4ᵇ            | 48 ± 0.13ᵃ                                    | PDH45+salt                         | 847 ± 1.7ᵇ            | 50 ± 0.12ᵃ                                    |

Data followed by the same letter in the same row are significantly not different at p < 0.05 as determined by Duncan’s multiple range test (n = 3).

**Figure 2.** Enzyme activity assay of soils (New Delhi, India). Variations of dehydrogenase (A) acid phosphatase (B), urease (C) and nitrate reductase (D) activities in: (1) WT control (–salt); (2) WT+salt; (3) T1-PDH 45+salt; (4) T1-PDH 45+salt (200 mM NaCl). Data are significantly not different at p < 0.05, n = 3.

Estimation of available carbon content of soil. One gram of soil sample was transferred to a 500 ml wide mouth flask and 10 ml 1 N K₂Cr₂O₇ was added to it and swirled to disperse the soil completely. Then 20 ml H₂SO₄ was added to it and allowed to stand for 30 min. Then 200 ml water was added followed by 3–4 drops of ferroin indicator. The solution was then titrated against 0.5 N ferrous ammonium sulfate (FAS).

Estimation of available nitrogen content of soil. Available nitrogen content of the soils was determined by modified Kjeldahl digestion method. A twenty gram soil sample was taken in a 800 ml Kjeldahl flask. Ten ml distilled water was added to resuspend the soil. Then, 100 ml 0.32% KMnO₄, a few glass beads, 2–3 ml paraffin liquid was added to it. Twenty ml (2%) boric acid mixed with indicator (bromo cresol green: methyl red::2:1) in a 250 ml conical flask was placed under receiver tube and was titrated against 0.02 N H₂SO₄ taken in burette until pink color started appearing.

Determination of available phosphorus in soil. The available phosphorus content of the soil was determined as explained by Page et al., (1982). Soil (2.5 g) was taken in a 100 ml conical flask, a pinch of Olsen’s reagent (0.5 M NaHCO₃) was added to it and the flask was shaken to thoroughly mix the ingredients. Five ml of filtered solution was taken in a 25 ml volumetric flask and 5 ml ammonium molybdate (0.5 N), 1 ml freshly prepared SnCl₂ (10 mM) solutions were added and the volume was made up to 25 ml by addition of distilled water. The optical density (OD) was measured at 660 nm.

Determination of available potassium and sodium in soil. The available potassium content of the soil was determined by method described by Jackson (1973). Soil (5 g) was taken in a 250 ml conical flask and 25 ml of 1 N CH₃COONH₄ solution was added to it. The flask was shaken for 30 min, the solution was filtered and the filtrate was diluted to 50 ml with 1 N CH₃COONH₄ solution and the data were measured by flame photometer (Bellstone) and the concentration was calculated by plotting the readings against the standard curve.

Sodium (Na) can be extracted with ammonium acetate solution in the same way as K, subsequently; Na in the extract can be determined by flame photometry. Certain elements, including Na, have the property that, when their salts are introduced into a flame, they emit light with a wavelength (color) specific to the element and of intensity proportional to the concentration.

Determination of available sulfur in soil. The available sulfur content of the soils was determined by method described by Chesin and Yien (1951). Twenty grams of soil sample was taken in a 250 ml conical flask, 100 ml monocalcium phosphate extracting solution (500 mg/l) was added to it and filtered. Ten ml filtrate was taken in a 25 ml volumetric flask, 2.5 ml 25% HNO₃, 2 ml acetic phosphoric acid, 0.5 ml BaSO₄ solution and 0.2 g of BaCl₂ crystals were added to it. Then the volume was made up to 25 ml and the optical density (OD) was measured at 440 nm.
Determination of calcium and magnesium from soil. The available magnesium and calcium contents of the soil were determined by method described by Jodral-segado (2006). The homogenized soil sample (300 mg) was placed in a 100-ml volumetric flask and mineralized by addition of 5 ml of concentrated HNO₃ and heated at 90°C for 45 min in a sand mineralization block. Five ml of 4:1 mixture of HNO₃ and HClO₄ was added to the solution and the heating was continued at 130°C for an additional 2 h until the sample was completely mineralized. Then the mixture was cooled and the resulting solution diluted to 25 ml with ultra pure water. A second dilution was prepared by taking different aliquots of the previous dissolution and diluting them with ultra pure water. In order to avoid the phosphate interference, 0.2 ml of LaCl₃ solution (10 mM) was added as a matrix modifier.

Calcium and magnesium determinations were performed by direct aspiration into the flame atomic absorption spectrophotometer (Perkin-Elmer 1100B double beam atomic absorption spectrophotometer, Perkin-Elmer Corp). The presence of matrix interference was observed only for calcium. Therefore, for this element the samples were analyzed by the standard addition method. All magnesium determinations were performed by the linear calibration method.

Isolation of rhizospheric bacteria. To obtain the isolated bacteria, serial dilution (10⁻⁴ dilution) prepared from 1 g soil sample was plated on nutrient agar media. Dissimilar colonies obtained on plates after an incubation time of 2–5 days at 35 ± 0.1°C were carefully picked. The individual colonies were checked for homogeneity under phase contrast microscope and the pure cultures were maintained both on slants of nutrient medium at 4 ± 0.1°C. The identification of the rhizospheric bacteria was performed as described earlier by Khan (2006).

Isolation of nematodes. The nematodes were extracted from PDH45 transgenic and non-transgenic (WT) rice plants in water suspension were uniformly placed over tissue paper supported by screen in petriplates, which were filled with water. After placing the sample, petriplates were covered with respective cap and incubated at 24 ± 2°C for 36 h. The nematodes present in soil were counted by transferring them to flat bottom partitioned counting dishes (36 squares) where the number of nematodes in 10 random squares were counted by observing under stereoscopic dissecting microscope (Nikon, 10–100× zoom). The averaged value of the count thus obtained was multiplied by 36 to get the total count of the nematodes.

Determination of soil dehydrogenase activity (DHA) activity. The soil dehydrogenase activity was evaluated as described earlier. Five grams of soil suspended in 5 ml of a TTC (Triphenyl Tetrazolium Chloride) solution (5 g TTC in 0.2 M Tris-HCl buffer, pH 7.4) was incubated at 37°C for 12 h. To stop the reaction, two drops of concentrated sulfuric acid were added followed by addition of toluene (5 ml). In order to extract TPF (Triphenylformazan), the mixture was shaken at 250 rpm for 30 min, followed by a 5 min centrifuging at 4,500 g. The color intensity of the supernatant thus obtained was measured at 492 nm by UV-Vis spectrophotometer (UV-1201, Shimadzu Corp).

Soil alkaline phosphatase activity. The alkaline phosphatase activity was measured by incubating 1 g soil with extraction solution (0.25 ml of toluene, 4 ml methylumbellifere (MUB) buffer (pH 11), 1 ml p-nitrophenolphosphate solution (in MUB buffer)) for 1 h. Following the incubation, 1 ml of CaCl₂ (0.5 M) and 4 ml of NaOH (0.5 M) were added to the flask and the contents were further incubated at 37°C for 1 h. The suspension was filtered (Whatmann no. 2 filter paper) and the absorbance of the filtrate was measured at 400 nm.

Figure 3. Enzyme activity assay of soils (Odisha, India). Variations of dehydrogenase (A) acid phosphatase (B), urease (C) and nitrate reductase (D) activities in: (1) WT control (−salt); (2) T±salt; (3) T1-PDH 45−salt; (4) T1-PDH 45+salt (200 mM NaCl). Data are significantly not different at p < 0.05, n = 3).
Soil urease activity. The urease activity in the soil was measured by hydrolyse urea in reaction mixture. Three biological and three technical replicates. The data was analyzed statistically and standard error was calculated. Analysis of variance (ANOVA) was performed on the data using SPSS (10.0 Inc) to determine least significant difference (LSD) for significant data to identify difference in the mean of the treatment.

Conclusion

In the present study it was observed that the PDH45 transgenic rice plants had no detectable adverse effects on the soil microbial community composition, physico-chemical properties and enzymatic activities of the soil rhizosphere as compared with their WT counterpart. The rhizospheric soil bacterial populations and enzyme activities revealed minor alterations among transgenic and non-transgenic rice plants and therefore, these studies have shown the possibility that there was no evidence to indicate any adverse effects of transgenic PDH45 on the native soil microflora.

Disclosure of Potentials of Interest

No potential conflicts of interest were disclosed.

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