Impact of Environmental Stress-Tolerant Transgenic Potato on Genotypic Diversity of Microbial Communities and Soil Enzyme Activities under Stress Conditions

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Transgenic crops able to tolerate environmental stress are being developed throughout the world. However, little data is available on the impact of environmental stress-tolerant transgenic crops on soil microorganisms and biochemistry. Recently developed transgenic potato plants carrying an environmental stress-related gene, DREB1A, with a stress-inducible promoter, are being evaluated for growth performance in greenhouses. In this study, we investigated microbial diversity and soil function to assess potential environmental risks of these transgenic potato lines. Genotypic diversity of the 16S-23S rRNA intergenic spacer region and activity levels of four enzymes were used as indicators of microbial genetic diversity and soil function, respectively. Salinity had a major effect on both bacterial (88–93%) and fungal (54–55%) diversity, while the transgene had a relatively small effect on genotypic structure (0–5%) based on the analysis of variance. However, a few genotypes appeared only in soils planted with the transgenic lines. Some enzyme activities were found to differ significantly between the transgenic and non-transgenic lines, although the results were not repeatable in the second trial. These results suggest that abiotic growth environments had a stronger impact on soil microorganisms and biochemistry than did plant genotypes.

Key words: biosafety, microbial diversity, soil enzyme activity, transgenic potato, environmental-stress tolerance

Despite the ongoing debate about the commercialization of transgenic crops, production areas of genetically modified (GM) crops are dramatically increasing33). Scientific risk assessments are vital for the commercial releases of living modified organisms to be used for food, feed, and processing (LMOs-FFP), while the final decision for a deliberate release into the environment is a complex issue that includes political, ethical, social, and risk-benefit balances, in addition to the scientific facts. After beginning any large cultivation of GM crops, monitoring must be carried out to observe the potential long-term effects31).

Potential risks associated with the production of transgenic crops, which can affect environments and ecosystems surrounding human activities, are: 1) replacement of traditional crops30); 2) genetic disturbance in wild-relative species or nearby non-transgenic production; and 3) unintended effects on non-target organisms, including soil microorganisms. The impact of plant genotypes on soil microbial communities has been a special focus in many environmental biosafety assessments, since soil microorganisms have direct interactive effects on plant growth and are fundamental groups in local ecosystems.

Changes in root exudation of plants may affect the structural and functional diversity of microorganisms17,27,29,48); soil types, including abiotic conditions3); plant types19); and plant physiological stage4), which also influence microbial communities. The sensitivity of microorganisms may give rise to different conclusions for environmental impact assessments of transgenic crop cultivation. Since growing conditions such as growing season may result in different outcomes, risk assessments should be taken with several ecological conditions to sort out the possible effects of transgenes and transgenic plants.

Assessments of genotypic diversity with molecular markers are often used to determine the changes in microbial diversity3,39), associated with transgenic crop cultivation4, 2,3,8). Molecular markers for soil microbes, such as Ribosomal Intergenic Spacer Analysis (RISA), are powerful tools for detecting changes in soil microorganism diversity and in community structure40) and have also been used to detect differences between transgenic and parental lines19). Soil conditions and functions associated with microbial functional diversity provide habitable niches for plants and other organisms, thereby regulating soil structure, supplying nutrients and controlling pests and diseases1. Altered microbial diversity can affect soil environments, though a small number of changes may not necessarily result in functional changes to the soil. When a species is absent, other species having the same niche and function may fill the vacancy and play a role in functional activities49). The assessment and monitoring of both diversity and function are thus crucial for developing appropriate regulatory and management strategies.

Measuring soil enzyme activity is an orthodox method for assessing changes in microbial activities. Although a variety of organisms and plants contribute to microbial activities, studying soil enzyme activity provides an overall view of changes in soil biological activities. Soil enzyme activities have been used for initial screening for potential environmental risk, which may be associated with transgenic plants25,28,42). Activity levels have been used in some assess-
ments of transgenic crops, and are sufficiently sensitive to detect major effects on soil environments\textsuperscript{2,47}. Thus, in this study, we used RISA to measure detailed changes in genotypic diversity and soil enzyme activities as indicators of soil function, in assessing the environmental-stress tolerant transgenic potato.

Plants undergo changes in the expression of stress-related genes in response to environmental stress. Environmental stress-tolerant crops, that employ foreign genes, are currently being developed in the US, the EU, Australia, Japan, and elsewhere. Transgenic potato lines of cv. Desiree, D163 and D164, have a transgene, DREBIA, that increases tolerance to various forms of environmental stress\textsuperscript{20}, including salt\textsuperscript{4}. DREBIA, encoding a binding factor for a dehydration-responsive element (DRE), was originally isolated from Arabidopsis\textsuperscript{24}, and in potato lines, was constructed to be expressed by a stress-inducible promoter, rd29A\textsuperscript{4}. The promoter expresses the gene when the plant faces environmental stress, and minimizes the plant’s fitness cost in non-stress situations\textsuperscript{39}. The transgenic potato, with stress-inducible expression of environmental stress tolerance, was developed to improve plant growth in saline environments without depressing growth in non-stress environments\textsuperscript{9}.

Despite the ranking by Hancock\textsuperscript{18} of environmental stress-tolerance genes as potentially having the highest relative fitness impact, few environment assessments have been reported for environmental stress-tolerant transgenic plants. Escaped environmental stress-tolerant plants may outperform in local ecosystems, especially when under the target stress, and the unintended impact on soil microorganisms must be assessed and quantified to develop environmental biosafety frameworks for stress-tolerant transgenic plants.

Here, we investigated potential impacts of environmental stress-tolerant crop production on soil microbial communities and soil functions in saline and non-saline conditions. The introduced gene, DREBIA, would have a higher expression level under saline conditions. The diversity of bacterial and fungal 16S-23S rRNA (ribosomal) intergeneric spacer regions was analyzed to evaluate the effects of saline treatment and the transgenic potato lines. Soil enzymatic activities were also evaluated for the impact of transgenic crop production on aspects of soil biological activity.

Materials and Methods

Experimental designs and soil

The cuttings of two transgenic lines of potato, D163 and D164, and their parental non-transgenic line (cv. Desiree, here referred to as DSN) were grown in a growth room until rooting occurred. Four weeks after cutting, young plants were individually planted in a 15 cm-diameter pot with approximately 1 L of soil, and moved to a greenhouse with 0.4 mm mesh screens on windows for environmental biosafety. The compost soil used in this study (0.96% total N, 0.31% P\textsubscript{2}O\textsubscript{5}, 0.46% K\textsubscript{2}O, C/N ratio 7.38, pH 6.1 at 20°C) was purchased commercially (Hana to Yasai no Baiyoudo, Kato Inc., Tochigi, Japan), and was mixed well before the experiments. No additional fertilizer was given during the experiments. The trial was repeated twice.

In the first trial, the plants were brought to the greenhouse from the growth room on March 19, 2007. The pots were arranged in a complete blocked design. The two treatments, tap (normal) water and saline (100 mM NaCl) water, had three replicates. Each replication consisted of two pots for each line. After 10 days of acclimation of young plants, approximately 0.5 L of 100 mM sodium chloride or tap water was applied for each treatment block when the surface of the soil in the pots became dry. The pots were free draining. We did not apply the saline water and tap water with the same frequency to avoid giving the plants too much water because 1) the plants treated with tap water usually used more water than those given saline water, and 2) the amount of water used by plants varies depending on daily climate in the greenhouse. Potato plants naturally prefer rather dry conditions and excess water can stress them.

The concentration of salt in each soil was determined at the end of the experiments. Ten milliliters of soil was added to 40 mL of distilled water in a 50 mL and centrifuge tube, centrifuged at 3000 rpm for 20 minutes. The supernatant obtained was filtered using No. 3 Whatman filter paper (Whatman, Maidstone, UK), and the amount of salt was measured using an electric conductivity, Salt6 Salinity Meter (Oakton Instruments, Vernon Hills, IL). The percentage of salt in the soils after harvest was as follows; 1.26% for DSN (SE 0.02; equivalent to 215.8±4.2 mM NaCl), 1.15% for D163 (SE 0.06; 196.9±10.4 mM) and 1.16% for D164 (SE 0.06; 198.3±10.4 mM), while less than the minimal detectable level (0.1%) was found in the soils from the control (tap water) treatments.

Soil was sampled from the rhizosphere and root/tuber area in the 10th week on June 6, 2007. Approximately 1 cm of top soil was removed first, and then the soil still attached to the plant removed from the pot was shaken free from the roots and sieved with a 2 mm sieve. The amount of soil collected from each pot was approximately 30 g. Equal amounts of soil from two replicates per line were bulked within blocks.

The second trial began on April 23, 2007, approximately one month after the beginning of the first trial. Rooted plants were prepared in the same way as in the first trial. Due to a limited number of rooted plants, three plants for each line were grown in the screened greenhouse in a complete randomized blocked design with two treatments. Soil from roots and the tuber area was sampled from each plant on July 9, 2007, and all samples were sieved with a 2 mm sieve. Soil DNA was extracted immediately after sampling, otherwise, soil samples were stored at 4°C for at most two weeks, until soil enzyme activities were measured.

DNA extraction and ribosomal intergenic spacer analysis (RISA)

DNA was extracted from 0.5 g of soil samples, using FastDNA SPIN Kit for soil (Qbiogene, Carlsbad, CA). The procedure followed the manufacturer’s protocol, except that skim milk (8 mg g\textsuperscript{-1} soil weight) was added to the extraction buffer before bead beating\textsuperscript{30}.

A spacer region of rRNA was amplified from soil DNA, using the primers ITS1/ITS4 for bacteria\textsuperscript{30} and 23SAC/312ST for fungi\textsuperscript{30}. These primers amplify the spacer region between the small (16S) and large (23S) subunits, and genotypic diversity is then interpreted from size differences. The PCR mixture (total 20 µL) contained 2U of Ex Taq DNA polymerase (Takara, Otsu, Japan), 1×PCR buffer (Takara), 0.5 µM each of the forward and reverse primers, 200 µM of each dNTP, and 10 ng of template DNA. The PCR conditions was as follows: 5 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and a final extension for 7 min at 72°C. The 5’ end of the forward primers was fluorescently labeled with 6-carboxyfluorescein (6-FAM). PCR products were run on 6% denaturing polyacrylamide gels with the GeneScan 2500 Red Dye (ROX) Size Standard (Applied Biosystems, Foster City, CA). The electrophoresis was run with 2000 V for 2.5 hours. Images of DNA fragments were visualized using Molecular Imager FX (Bio-Rad Laboratories Inc., Hercules, CA). The visualized bands were scored using Quantity One software (Bio-Rad Laboratories, Inc.).
Soil Enzyme Activities

All soil enzyme activities were measured following the method of Tabatabai[1]. Soil urease activity was measured using 5 g of fresh soil incubated with urea, by determining NH₃-N using steam distillation[2]. For determining the activity levels of alkaline phosphomonoesterases, 1 g of fresh soil was incubated with p-nitrophenyl phosphate hexahydrate, in modified universal buffer[3][4][5]. Arylsulfatase activity was measured using 1 g of fresh soil incubated with potassium p-nitrophenyl sulfate as a substrate[6]. β-glucosidase activity was measured using 1 g of fresh soil incubated with p-nitrophenyl-β-D-glucoside[7]. With the alkaline phosphomonoesterase, arylsulfatase, and β-glucosidase, the p-nitrophenol produced by the enzymes after incubation with each substrate was determined as equivalent to 0.05 M disodium p-nitrophenyl phosphate. The color intensity of the p-nitrophenol was measured with a spectrophotometer at a wavelength of 420 nm.

Data analysis

Genotypic diversity in bacterial and fungal communities, measured with RISA: Each replicated experiment was analyzed separately to avoid misinterpretations due to the inability to match alleles of the same molecular size across the gels. The effects of treatments and plant genotypes on the microbial community structure were tested by analysis of molecular variance (AMOVA)[8] using GenAlex[9]. Genotypic structural differentiation was estimated based on genotypic variance, with 1000 permutations[10]. The genotypic structural differentiation among plant genotypes within treatments was denoted as Fst in this study. The clusters, reflecting genetic relationships between soil samples from each line, were constructed with the UPGMA method.

Soil enzyme activity: Measurements were weighted according to the concentration per 1 g of dried soil. Analysis of variance (ANOVA) was performed to detect the effects of the treatments and plant genotypes using R version 2.5.1. Differences in enzyme activities between the soil with transgenic genotypes, and the soil with non-transgenic plant genotypes was tested with the Student t test.

Results

Genetic diversity of the microbial community

A total of 36 and 34 genotypes of bacteria were scored in the first and second experiments, respectively. Twelve and ten genotypes of fungi were also scored (with confidence) in the first and second experiments, respectively. A few plant line-specific genotypes were present in the bacterial and fungal communities (Table 1; Fig. 1), although no line-specific genotypes were consistently found in the non-transgenic or transgenic lines.

The AMOVA indicated that salinity had significant effects on the variation in microbial communities (Table 2; p=0.01 for both trials in bacteria and fungi). In both replicated experiments, the bacterial communities were severely affected by salinity (88 to 93% of the total variation), but little affected by plant genotype (2 to 3%). The fungal communities, on the other hand, were moderately affected by the saline conditions (54 to 55% of the total variation), and showed relatively high levels of variation within the plant genotypes (40 to 46%). Genotypic structural differentiation among plant genotypes within each treatment was not statistically significant in either bacteria or fungi in the two trials (Table 2).

The cluster analysis revealed that the samples in saline or normal conditions tended to group together, suggesting a similar genotypic composition in the same environments in both bacteria (Fig. 2) and fungi (Fig. 3). However, a major cluster was formed each for normal and saline conditions in the bacterial RISA, while several sub-clusters were observed in the fungal RISA, showing weaker trends under saline conditions in the fungal community.

Soil enzymatic activities

Soil enzyme activities were generally weaker following the saline treatment (100 mM NaCl) than the tap water treat...
Table 2. AMOVA table for bacterial and fungal RISA

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Comp. of variance</th>
<th>Genotypic structural differentiation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Est. variance (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial RISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between treatments</td>
<td>1</td>
<td>54.06</td>
<td>54.06</td>
<td>5.86</td>
<td>88%</td>
<td>F_{\text{IT}}=0.88</td>
</tr>
<tr>
<td>Among plant genotypes</td>
<td>4</td>
<td>5.11</td>
<td>1.28</td>
<td>0.22</td>
<td>3%</td>
<td>F_{\text{GT}}=0.27</td>
</tr>
<tr>
<td>Plants within plant genotypes</td>
<td>12</td>
<td>7.33</td>
<td>0.61</td>
<td>0.61</td>
<td>9%</td>
<td>F_{\text{GT}}=0.91</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>66.5</td>
<td>55.94</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between treatments</td>
<td>1</td>
<td>65.17</td>
<td>65.17</td>
<td>7.15</td>
<td>93%</td>
<td>F_{\text{IT}}=0.93</td>
</tr>
<tr>
<td>Among plant genotypes</td>
<td>4</td>
<td>3.33</td>
<td>0.83</td>
<td>0.13</td>
<td>2%</td>
<td>F_{\text{GT}}=0.23</td>
</tr>
<tr>
<td>Plants within plant genotypes</td>
<td>12</td>
<td>5.33</td>
<td>0.44</td>
<td>0.44</td>
<td>6%</td>
<td>F_{\text{GT}}=0.94</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>73.83</td>
<td>66.44</td>
<td>7.72</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal RISA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between treatments</td>
<td>1</td>
<td>13.78</td>
<td>13.78</td>
<td>1.42</td>
<td>54%</td>
<td>F_{\text{IT}}=0.56</td>
</tr>
<tr>
<td>Among plant genotypes</td>
<td>4</td>
<td>3.78</td>
<td>0.94</td>
<td>0.00</td>
<td>0%</td>
<td>F_{\text{GT}}=0.00</td>
</tr>
<tr>
<td>Plants within plant genotypes</td>
<td>12</td>
<td>14.67</td>
<td>1.22</td>
<td>1.22</td>
<td>46%</td>
<td>F_{\text{GT}}=0.52</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>32.22</td>
<td>15.94</td>
<td>2.56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Trial</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between treatments</td>
<td>1</td>
<td>8.44</td>
<td>8.44</td>
<td>0.84</td>
<td>55%</td>
<td>F_{\text{IT}}=0.55</td>
</tr>
<tr>
<td>Among plant genotypes</td>
<td>4</td>
<td>3.33</td>
<td>0.83</td>
<td>0.07</td>
<td>5%</td>
<td>F_{\text{GT}}=0.11</td>
</tr>
<tr>
<td>Plants within plant genotypes</td>
<td>12</td>
<td>7.33</td>
<td>0.61</td>
<td>0.61</td>
<td>40%</td>
<td>F_{\text{GT}}=0.60</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>19.11</td>
<td>9.89</td>
<td>1.53</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sources of variance were between treatments (normal vs. salinity), and among plant genotypes. Genotypic structural differentiation was estimated based on genotypic variance with 1000 permutations, F_{\text{IT}}: differentiation between treatments, F_{\text{GT}}: differentiation among plant genotypes within a treatment, F_{\text{GC}}: differentiation within plant genotypes.

Fig. 2. Clustering analysis using the UPGMA method for bacterial genotypic variation based on RISA from the first (a) and second (b) trial. N: normal conditions, S: saline conditions, DSN: parental genotype cv. Desiree, D163 and D164: transgenic lines with DREB1A. The Dice coefficient was estimated as a Similarity Index. The scale at the top of each dendrogram is of the similarity coefficients.

Fig. 3. Clustering analysis using the UPGMA method for fungal genotypic variation based on RISA from the first (a) and second (b) trial. N: normal conditions, S: saline conditions, DSN: parental genotype cv. Desiree, D163 and D164: transgenic lines with DREB1A. The Dice coefficient was estimated as a Similarity Index. The scale at the top of each dendrogram is of the similarity coefficients.
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![Graphs showing soil enzyme activities](image)

**Fig. 4.** Soil enzyme activities from the first and second trials: urease (a), arylsulfatase (b), alkaline phosphomonoesterase (c), and β-glucosidase (d). Open bar indicates the normal environment, and shaded bar indicates the saline environment. Error bars indicate standard error estimated from three replicated plant genotypes. Each measurement was repeated three times and the averaged values were analyzed.

**Table 3.** Probability (p-value) according to the pair-wise Student t test of differences in soil enzyme activities between transgenic and non-transgenic lines, and between treatments

<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Urease</th>
<th>Phosphomonoesterase</th>
<th>Arylsulfatase</th>
<th>Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>DSN</td>
<td>D163</td>
<td>0.393</td>
<td>0.173</td>
<td>0.217</td>
<td>0.758</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>DSN</td>
<td>D164</td>
<td>0.952</td>
<td>0.128</td>
<td>0.377</td>
<td>0.588</td>
</tr>
<tr>
<td></td>
<td>overall</td>
<td>Normal</td>
<td>Salinity</td>
<td>0.001</td>
<td>0.815</td>
<td>0.453</td>
<td>0.527</td>
</tr>
<tr>
<td>2</td>
<td>Normal</td>
<td>DSN</td>
<td>D163</td>
<td>0.406</td>
<td>0.725</td>
<td>0.888</td>
<td>0.447</td>
</tr>
<tr>
<td></td>
<td>Salinity</td>
<td>DSN</td>
<td>D164</td>
<td>0.424</td>
<td>0.443</td>
<td>0.750</td>
<td>0.978</td>
</tr>
<tr>
<td></td>
<td>overall</td>
<td>Normal</td>
<td>Salinity</td>
<td>0.003</td>
<td>0.125</td>
<td>0.013</td>
<td>0.178</td>
</tr>
</tbody>
</table>

Statistical significance is indicated in bold when p-value was p<0.05.

ment (Fig. 4), though only the urease activity was significantly affected in both trials (Table 3).

No significant difference in soil enzyme activities was found between the transgenic lines, D163 and D164, and their parental non-transgenic line, DSN, except for a difference in arylsulfatase activity (p=0.053 in the first trial) and in alkaline phosphomonoesterase activity (p=0.054 in the second trial) between DSN and D164 in the saline environment (Table 3). These differences, however, were not seen in either of the replicated trials.

**Discussion**

**Impacts of soil salinity on soil environments**

The bacterial community structure, in particular, was strongly affected by salinity. A range of bacterial species may have evolved to tolerate highly saline environments\(^{(1)}\). A shift was observed between non-saline and saline soils, perhaps because more saline-tolerant bacterial species populated in saline soil than did saline-susceptible species. Thus, community structure was largely affected by soil conditions relative to the transgene or plant genotype. On the other hand, the fungal communities were less affected by saline
conditions than the bacterial communities in this study, and had more variation among the individual pots. Considering that the bacterial communities showed very similar patterns among the pots within treatments, the variation in the fungal communities was perhaps caused by heterogeneity and differentiation in colonization speed during the three-month growth period in the greenhouse.

The number of soil microorganisms existing in the environment is enormous. Some share the same niche and have the same functions\(^4\), and because of the redundancy of microbial functional groups, the presence or absence of a given species may not necessarily result in changes to the ecosystem such as soil biological activities. In our study, only the soil urease activity consistently differed between the treatments, though bacterial as well as fungal genotypic structure was severely affected by the treatments. Although not all enzyme activities are derived from bacteria and fungi, the results suggest that the presence of species having different niches, but the same soil functions, maintains a similar overall pattern of activity.

**Impacts of the transgene on soil environments**

The transgenic lines, D163 and D164, have a stress-inducible transgene, which shows a high level of expression in saline, compared to control (non-saline) environments\(^4\). If the transgene is expressed at higher levels under saline conditions and influences microbial diversity directly or indirectly, the impact may be more pronounced in saline conditions. The analysis of microbial genotypic diversity suggested that the transgene and the genotypes observed had less of an impact than the saline treatment, even in saline soils.

No significant differences were seen in genotypic structure between the soils with transgenic and non-transgenic lines that could be attributed to the transgene. However, at the same time, a few genotypes were present only in the transgenic lines, though in only one or two of three replicated plant soils (Fig. 1). Beside effects of the transgene itself, such a difference may be caused by changes in root exudate, rates of root death in saline environments, and plant water or nutrient uptake due to the saline tolerance of the transgenic plants. Interpreting minor changes in genotypic structure is difficult, because minor changes in species (or genotypic) composition could have a significant impact on the soil environment, but the occurrence of a few genotypes is not enough to detect statistical significance in such analyses. Additional assessments and monitoring looking at some soil functional aspects are required to address the ultimate effects of those changes.

The activities of urease and \(\beta\)-glucosidase, used here as indicators for soil functions, did not show repeatable significant differences between the transgenic and non-transgenic plant soils (Table 3). However, in one of the repeated trials, under saline conditions, significant differences in the activities of alkaline phosphomonoesterases and arylsulfatase were seen between the transgenic (D164) and non-transgenic (DSN) plants (Table 3). Arylsulfatase has been found in plants, animals, and microorganisms\(^2\), while alkaline phosphomonoesterase is only derived from soil microorganisms (higher plants are devoid of this enzyme)\(^2\). Thus, the changes in alkaline phosphomonoesterase activity are solely due to changes in microbial activities. The samples for the second trial were collected on July 9th while the first trial ended on June 6th in the greenhouse. Differences in temperature and other environmental factors might have affected the plant physiology and microbial activities. Differences in the environments may also influence expression levels of the transgene. Greenhouse conditions can generate stress for potatoes such as high temperatures, which could have activated the gene in the absence of saline stress (Shimazaki et al., unpublished data).

Because crops grow in fields, they face abiotic and biotic environmental fluctuations. This may cause different experimental outcomes during assessments and monitoring. For example, for transgenes that confer herbicidal tolerance, studies have found no evidence for unintended effects on soil microbes\(^3,5,7,10\). In other studies, however, changes were detected in the microbial diversity of soils planted with transgenic crops\(^10,40,41\), that were at least temporary\(^11\). In environmental stress-tolerant transgenic crops, not only transgenes but also the plant itself could respond to various forms of environmental stress. Considering the biological and ecological variation in fields, assessments and long-term post-implementation monitoring will lead to the proper management of transgenic crops\(^3\).

In conclusion, the results suggested the impact of the transgene not to be strong enough to outweigh the effects of salinity itself and seasonal variation on soil microorganisms and soil biochemistry. However, it should be noted that the detected changes in microbial genotypes and soil enzyme activity tended to be seen in the saline condition, where the expression level of the transgene would be higher. Further investigation, including of the expression levels of the transgene with time, is necessary to ensure whether these changes persist over time and space for better management of the transgenic potato.

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**References**

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