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BIOSAFETY ASSESSMENT OF GENETICALLY ENGINEERED WHEAT (*Triticum aestivum* L.) UNDER GREENHOUSE CONDITIONS

Ashtar Khan[1], Ghulam Muhammad Ali[2], Shaukat Ali[2], Muhammad Amir Zia[2], Sohail Ahmad Jan[2] and Um-E-Robab[3*]

[1]Bio- Resources Conservation Institute (BCI), National Agricultural Research Centre (NARC) Islamabad, Pakistan; [2]National Institute for Genomics and Advanced Biotechnology (NIGAB), National Agricultural Research Centre(NARC) Islamabad, Pakistan; [3]Pakistan Institute of Nuclear Science and Technology, (PINSTECH) Islamabad, Pakistan
[*]Corresponding author's e-mail: ume_robab@yahoo.com

The enormous development in gene transfer technology made it possible to evolve new cultivars with valuable agronomic traits but escape of transgenes from genetically modified crops is one of the major concerns for biosafety. This study was aimed to investigate the potential flow of rice chitinase gene (*RCG3*) from transgenic wheat. Soil nutritional analysis was done through AB-DTPA method, while soil bacteria were isolated and identified based on 16S rRNA gene sequencing. The transgenic plant showed the presence of *RCG3* gene after PCR analysis while it was absent in control plants. The same gene was absent in both soil samples collected from transgenic and non-transgenic plants. Soil nutritional analysis showed KNO_3 (1.53 mg/kg) and phosphorous contents (1.35 mg/kg) in transgenic wheat soil as compared to their counterparts that gave 3.01 and 2.51 mg/kg KNO_3 and phosphorous contents, respectively. Bacterial colonies count showed the maximum number in transgenic wheat rhizosphere ($2x10^4$ CFU/g of a total replication in dilution factor 10^{-2}). Results showed that both strains of transgenic and control rhizospheric wheat were from the same group (97% sequence similarity) showing the same common ancestor. These results clearly demonstrated that neither vertical nor horizontal gene flow occurred from transgenic wheat.
Keywords: Gene flow, phylogenetic analysis, *RCG3* gene, rhizosphere, *16S rRNA* gene, *Triticum aestivum* L.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a staple food for a great deal of population in the world, especially in Asia. It is susceptible to many diseases particularly fungal diseases which greatly reduce its potential yield. Such diseases are usually managed with the application of proper fungicides. However, persistent and excessive use of agrichemicals could have adverse effects on environment (Gullino *et al*., 2000). To combat this limitation, scientists have relied on selected gene transfer into wheat with valuable agronomic characteristics (Anthony *et al.,* 2001; Goyal and Prasad, 2010). The plant chitinase gene has been transferred into different crop plants, to develop resistance against fungal diseases and to reduce the use of environmentally damaging agrichemicals (Salami *et al.,* 2008). Chitinases are proteins which are used for protection of plants against fungal diseases. Chitin is a basic component of the cell wallsof all fungal cells whereas;chitinases are digestive enzymes and have the capability to rupture N-acetylglucosamine polymer chitin of fungal cell wall (Ano *et al.,* 2003). Therefore, researchers have transformed diverse plant chitinases such as rice chitinase gene (*RCG*) (Hong *et al.,* 2005), wild spinach chitinase gene (Hirai *et al.,* 2004), insect chitinase gene (Saguez *et al.,* 2005) against fungal attacks in a range of plants.

Ever increasing production and use of transgenic plants is raising concerns regarding bio-safety issues worldwide (Crawley *et al*., 2001; Ellstrand, 2001; Prakash, 2001; Snow, 2002). Though these applications may provide rapid solution to the concerned problem however, they may also create hazardous conditions to agriculture, environment as well as human and other living population such as microorganisms (Anthony *et al.,* 2001). The possible environmental risks linked with transgene escape are the leading among these concerns (Amand *et al.,* 2000; Halfhill *et al.,* 2001; Lavigne *et al.,* 2002). Gene flow is a natural process that results from transfer of gene between related and unrelated species, when crops are hybridized with the population of the adjacent area. The gene flow mechanism is under the focus of risk assessment and results in environmental pollution and destruction of plant genetic resources or wild relatives as well as production of resistant rhizospheric microbes (Eastman and Sweet, 2002; Dunfield and Germida, 2004). There is also possibility that incorporation of a certain gene into a plant might create a new allergen or cause hypersensitive reaction in susceptible individuals (Losey *et al*., 1999). Among organisms to be affected, soil bacteria are one of the closely related organisms vulnerable to transgenic plant by gene transfer mechanism.

Gene transfer could either be horizontal/lateral or vertical. Horizontal gene transfer (HGT) is the movement or transfer of genetic material (DNA) directly (in a manner other than traditional reproduction) to an organism or a living cell. Such gene transfer occurs in organisms that are not parent and offspring rather unrelated and often different species. HGT is the most common method by which unicellular organisms particularly bacteria acquire new genetic material. In contrast, during cell division, vertical gene transfer is the transmission of genetic material from mother cell to daughter cell as it occurs in reproduction. Release and commercialization of genetically modified crops need to be passed through biosafety evaluation to avoid any possible adverse effects on other related or unrelated organisms and ecosystem. Regardless of the international debate on the commercialization of genetically engineered crops, countries have agreed on the Cartagena Protocol about various biosafety issues such as implementation of guidelines, trade policies frame work and management of GM products around the world.

This research work was designed to investigate the effect of transgenic wheat (having rice chitinase gene *RCG3*) on soil bacteria as well as on conventional wheat crop. The purpose of this study was to determine the risk assessment of vertical gene flow into non-GM plants of wheat growing in adjacent location. Moreover, horizontal gene flow in the rhizospheric soil of GM and non- GM wheat plants was also assessed.

MATERIALS AND METHODS

This study was performed at National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC, Islamabad, Pakistan to evaluate transgenic wheat lines for biosafety concerns.

***Plant material and soil sampling*:** Transgenic wheat (Chakwal-93) harboring rice chitinase gene (*RCG3*) was obtained from NIGAB and evaluated for vertical and horizontal gene flows into non-transgenic plant (control) and other soil containing bacteria. To study the biosafety response of wheat, both transgenic and control seeds were transferred to soil filled pots. The fresh and clean two to three seeds were sown in separate transgenic containment at 25 ± 5°C. After seed germination, leaf samples from transgenic and control wheat plants were taken for the confirmation of *RCG3* gene. After maturity, seeds from both transgenic and control wheat were collected and stored for further *RCG3* confirmation. At the same time soil samples from both transgenic and control pots were collected for the presence/absence of horizontal gene flow from plants to soil. Surface rhizospheric soil was taken for this purpose.

***Nucleic acid extraction and PCR analyses*:** To detect the presence of *RCG3* gene, genomic DNA was extracted from leaf and seed samples of transgenic and control plants using CTAB method as described by Sambrook and Russel (2001). Similarly, the DNA was extracted from soil samples of both transgenic and control plants by using protocol of Sambrook *et al*. (1989). PCR analysis was used to confirm the presence of chitinase gene in wheat genome. The *RCG*-3 specific forward and reverse primers were used for the amplification of 750 bp fragment of transformed gene in wheat. The sequence of the primers was: RCG3 F 5'-CTCCACCTCCGATTACTGC-3', while RCG3 R 5'-GTAGG GCC TCTGGTTGTAGC-3'.

The complete PCR profile of *RCG3* gene amplification was: pre-denaturation was done at94ºC for 5 mins followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 52°Cfor45 sec, initial extension at 72°C for 90 sec and final extension was done at 72°Cfor 20 min. Same protocol for PCR reaction conditions was adopted for soil samples (soil microflora) of both transgenic and control rhizospheric wheat. PCR product was run on 1% agarose gel and visualized under UV light condition by using gel documentation system.

***Soil nutritional analyses*:** In this experiment soil samples were collected from the cultivated pots (depth of 15 cm). The collected soil samples were thoroughly ground, homogenized and sifted with the help of 2 mm sieve. The prepared soil was analyzed for its physical and nutritional distinctiveness. A composite sample of this soil was air dried and ground with the help of mortar and pestle (Peterson and Calvin, 1986). Chemical and nutritional analyses *i.e.* soil pH, electrical conductivity; nitrate- K, available Phosphorus and Potassium were determined in the soil of both transgenic and control plants. Soil electrical conductivity was measured through electric conductivity meter.

***Isolation, pre-culturing and counting of bacteria from rhizospheric soil*:** Isolated soils were picked with the help of sterilized loop and streaked on LB agar plates. The streaked plates having media were incubated at 28°C. The growth of isolated bacterial colonies was appeared after 3 days of incubation. These colonies were identified as having milky color. From the LB agar plate, the microbial growth was picked through sterilized loop and subsequently streaked on the media plate and then it was properly labeled. To count bacterial colonies, diverse soil samples were collected from transgenic and control wheat rhizosphere. Soil samples were taken from a depth of 0-15 cm and subsequently stored at room temperature for further investigation. Isolation of bacteria was scheduled from every sample through dilution plating count method. 10 folds of serial dilutions were prepared from soil suspension. About 10 ml of every dilution was spread on agar plate having LB media and stored in incubator at 27–30°C for 24 hrs. The colonies appeared on plates after 24 hrs were counted and tagged.

***Identification of bacterial isolates*:** All the isolated bacterial strains were identified by using 16S rRNA gene sequencing. The universal forward and reverse primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') matched to *E. coli* 16S rRNA

positions 8-28 and 1541-1522 were used for the amplification of target 16s rRNA gene segment by using protocol of Ahmed *et al*. (2007).

***Nucleic acid extraction and 16S rRNA amplification*:** The DNA template was prepared by suspending the bacterial cells in sterilized PCR strips (MBP, USA) containing 20 µl of Tris-EDTA (TE) buffer. Few well-isolated bacterial colonies were gently suspended in TE buffer. For 10 minutes the suspended cells in TE were heated at 95°C in PCR machine and then centrifuged (3000 rpm) for 6 minutes. The supernatant containing bacterial DNA was used as a template for the amplification of 16S rRNA gene. The PCR amplification of 16S rRNA gene was carried out in a 50 µl reaction mixture. The reaction mixture was prepared by mixing 1 µl of template DNA, 25 µl of Premix Ex-Taq (TAKARA, Japan), 2 µl of each forward and reverse primers (9F and 1510R; 10 pmol μl^{-1}) and 20 µl of PCR quality grade water. Amplification was performed in a thermocycler (ABI Verity, USA). The amplification of 16S rRNA gene was confirmed by 0.8% gel electrophoresis.

***Identification, purification and sequencing of amplified 16S rRNA gene*:** Purification of Amplified PCR products 16S rRNA genes were made by PCR Purification kit (Invitrogen, USA). The purified PCR product was sequenced using universal (27F and 1492R) universal primers by commercial service of Macrogen, Korea (http://dna.macrogen.com/eng). Bio-Edit Software package (version 7.0.8) was used for sequence editing and contigs assembly. A contig sequence of 16S rRNA gene by one primer was trimmed at both ends to obtain a good quality sequence keeping in view the peaks of chromatographs of sequence results. The sequences of both contigs obtained with forward and reverse primers were assembled in Contig Assembly Program (CAP) contained in Bio-Edit Software package. The bacterial strains were identified on EzTaxon Server 2.1 (http://eztaxon-e.ezbiocloud.net) based on full-length and partial sequences of 16S rRNA gene by similarity with validly published bacterial species. The sequences were submitted to DNA Databank of Japan (DDBJ) and accession number of 16S rRNA gene of each strain was obtained.

***Phylogenetic analysis*:** For phylogenetic analysis, the sequences of closely related validly published species were retrieved from the Ez-Taxon Server. The sequence of isolated strains and closely related species were aligned in CLUSTAL X (version 2.0.11) Software. The gaps and ambiguous nucleotides were removed using Bio-edit sequence Alignment Editor. The unambiguously aligned sequences data were used to generate evolutionary distances and the phylogenetic trees were constructed using MEGA-5 Program (Tamura *et al*., 2011) by Neighbor-joining (Saitou and Nei, 1987). The stability of the phylogenetic tree was assessed by calculating bootstrap value by performing 1000 re-sampling of sequences data for tree topology.

***Statistical analysis*:** All these experiments were performed in three replicates using Randomized Complete Block Design (RCBD) with pot to pot distance of 20 cm in the wheat growing season 2012.

RESULTS

***Assessment of horizontal and vertical RCG3 gene flow*:**Total genomic DNA was extracted from transgenic and control wheat plants by CTAB method devised by Sambroke and Russel, 2001 (Fig. 1a-c). In transgenic wheat, the amplified product of chitinase gene was 750 bp, while no band appeared in control wheat plants (Fig. 1d). To check horizontal gene flow, we tested RCG3 gene in soil bacteria. To detect the presence of RCG3 gene, soil DNA (soil rhizosphere) was extracted from transgenic and control rhizospheric soil through CTAB method. The same process of PCR reaction conditions was also adopted for soil samples of both the transgenic and control rhizospheric wheat. The results revealed that there were no expected bands observed in transgenic soil samples. The same case was also true for control rhizospheric soil samples (Fig. 1e). The main theme of this experiment was to assess the biosafety concerns of transgenic wheat. We successfully evaluated the *RCG3* gene flow both horizontally and vertically and found no gene flow from transgenic wheat.

***Assessment of soil for nutritional contents*:** In present study soil samples of both transgenic and control wheat were tested. The results revealed that higher levels of electrical conductivity (0.18 dS/m) were obtained in transgenic soil samples than that of control soil samples (0.15 dS/m). Similarly, more phosphorous contents (2.8 mg/kg) were recorded in soil samples of transgenic plants as compared to soil samples of control plants (2.51 mg/kg) (Table 1). Likewise, higher KNO_3 level (1.53 mg/kg) were recorded in transgenic soil sample as compared to soil sample of control wheat plants that gave 1.35 mg/kg KNO_3 (Table 1). The results of soil pH showed that transgenic sample exhibited more basic pH (8.26 values) as compared to control sample (pH 8.16) (Table 1).

***Assessment of the number of bacterial colonies*:** Results of colony counting showed that more colonies ($2x10^4$CFU/g) were obtained in transgenic wheat rhizosphere which were of total replication in dilution factor 10^{-2} followed by dilution factor 10^{-3} and 10^{-4} which were too few to count. Similarly, in control wheat rhizosphere, the maximum colonies ($1.8x10^4$ CFU/g) were recorded on nutrient agar plate in dilution factor 10^{-2} with a total of three replications followed by dilution factor 10^{-3} and 10^{-4} (Table 2).

Table 1. Assessment of transgenic and NT wheat based on soil nutrients.

Soil characteristics	Non-transgenic wheat plants	Transgenic wheat plants
Electrical conductivity (dS/m)	0.15[d]	0.18[d]
Phosphorous contents (mg/kg)	2.51[b]	2.8[b]
KNO_3 (mg/kg)	1.35[c]	1.53[c]
pH	8.16[a]	8.26[a]

Phosphorous and potassium nitrate were extracted by following the procedure of AB-DTPA. AB-DTPA denotes ammonium bicarbonate-DTPA (diethylene triaminepenta acetic acid) and measured in mg/kg. Soil electrical conductivity was measured through electric conductivity meter. Statistically no significant differences were recorded among transgenic and control plants.

Table 2. Assessment of number of colonies of soil bacteria isolated from the rizhosphere of both transgenic and non-transgenic plants.

Dilution factor	Non-transgenic plants		Transgenic plants	
	No. of colonies per NA plate	CFU/g Soil	No. of colonies per NA plate	CFU/g Soil
10^{-2}	94	1.8×10^4	100	2.0×10^4
10^{-3}	TFTC	TFTC	TFTC	TFTC
10^{-4}	TFTC	TFTC	TFTC	TFTC

NA denotes nutrient agar; TFTC denotes too few to count; CFU denotes colony forming unit

***Assessment of RCG3 gene flow by phylogenetic analysis*:** The isolated strains of rhizospheric bacteria from transgenic and control wheat was further subjected to phylogenetic analysis. Result showed that strains isolated from transgenic and control rhizospheric wheat soil fell into the same group showing the same ancestor (belongs to the genus *Bacillus*). Both strains i.e. NCCP-781 (isolated from transgenic rhizosphere) and NCCP-782 (isolated from control rhizosphere) were strains of *Bacillus tequilensis* as shown in (Table 3-6; Fig. 1f & g; Fig. 2).

Similarly, the strains NCCP-783 (isolated from transgenic rhizosphere) and NCCP-784 and NCCP-787 (isolated from control rhizosphere) were the strains of *Bacillus anthracis*. Results clearly demonstrated that isolates of transgenic and control rhizosphere shared the same strains with no difference in micro flora. NCCP-781 showed more than 97% similarity with already identified strains *Bacillus tequilensis* (99.82%), *Bacillus subtilis* sub sp. *Subtilis* (99.75%), *Brevibacteriumhalotolerans* (99.66%), *Bacillus subtilis* subsp. (*spizizenii*) (99.66%), *Bacillus mojavensis*) (99.66%), *Bacillus siamensis* (99.49%), *Bacillus methylotrophicus* (99.41%), *Bacillus vallismortis*) (99.32%), *Bacillus amyloliquefaciens* subsp. *Plantarum* (99.24%), *Bacillus amyloliquefaciens* subsp. *Amyloliquefaciens* (99.24%), *Bacillus atrophaeus* (99.24%), *Bacillus licheniformis* (98.13%) and *Bacillus aerius* (97.87%) (Tables 3& 4; Fig. 2).

Table 3. Assessment of resemblance of strains showing more than 98% similarities to NCCP 781 (Transgenic) by phylogenetic analysis.

S. No	Related strains	Accession No.	Identity (%)
1	*Bacillus tequilensis* 10b(T)	HQ223107	99.83
2	*Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28(T)	EU138467	99.82
3	*Bacillus subtilis subsp. subtilis* NCIB 3610(T)	ABQL01000001	99.75
4	*Brevi bacterium halotolerans* DSM 8802(T)	AM747812	99.66
5	*Bacillus subtilis subsp. spizizenii* NRRL B-23049(T)	CP002905	99.66
6	*Bacillus mojavensis* RO-H-1(T)	JH600280	99.66
7	*Bacillus siamensis* KCTC 13613(T)	AJVF010043	99.49
8	*Bacillus methylotrophicus* CBMB205(T)	EU194897	99.49
9	*Bacillus vallismortis* DV1-F-3(T)	JH600273	99.41
10	*Bacillus amyloliquefaciens* subsp. *plantarum* FZB42(T)	CP000560	99.32
11	*Bacillus amyloliquefaciens* subsp. *amyloliquefaciens* DSM 7(T)	FN597644	99.24
12	*Bacillus licheniformis* ATCC 14580(T)	AE017333	99.24
13	*Bacillus atrophaeus* JCM 9070(T)	AB021181	98.13
14	*Bacillus aerius* 24K(T)	AJ831843	98.87

Table 4. Assessment of resemblance of strains showing more than 97% similarities to NCCP 781 (Transgenic) by phylogenetic analysis.

S. No	Related strains	Accession No.	Identity (%)
1	*Bacillus anthracis* ATCC 14578(T)	HQ223107	99.83
2	*Bacillus pseudomycoides* DSM 12442(T)	EU138467	99.82
3	*Bacillus mycoides* ATCC 6462(T)	ABQL01000001	99.75
4	*Bacillus anthracis* Ames	AM747812	99.66
5	*Bacillus cereus* ATCC 14579(T)	CP002905	99.66
6	*Bacillus thuringiensis* ATCC 10792(T)	JH600280	99.66
7	*Bacillus mycoides* DSM 2048(T)	AJVF010043	99.49
8	*Bacillus weihenstephanensis* WSBC 10204(T)	EU194897	99.49
9	*Bacillus weihenstephanensis* KBAB4	JH600273	99.41
10	*Bacillus gaemokensis* BL3-6 KCTC 3318(T)	CP000560	99.32
11	*Bacillus cytotoxicus* NVH 391-98(T)	FN597644	99.24
12	*Bacillus manliponensis* BL4-6(T)	AE017333	99.24

Table 5. Assessment of resemblance of strains showing more than 94% similarities to NCCP 782 (Non-Transgenic) by phylogenetic analysis

S. No	Related strains	Accession No.	(%)
1	*Bacillus subtilis* subsp. *inaquosorum* BGSC 3A28(T)	EU138467	97.95
2	*Bacillus tequilensis* 10b(T)	HQ223107	95.34
3	*Bacillus subtilis* subsp. *subtilis* NCIB 3610(T)	ABQL01000001	95.27
4	*Brevibacterium halotolerans* DSM 8802(T)	AM747812	95.2
5	*Bacillus subtilis* subsp. *spizizenii* NRRL B-23049(T)	CP002905	95.2
6	*Bacillus mojavensis* RO-H-1(T)	JH600280	95.12
7	*Bacillus siamensis* KCTC 13613(T)	AJVF01000043	95.05
8	*Bacillus vallismortis* DV1-F-3(T)	JH600273	94.98
9	*Bacillus myloliquefaciens* subsp. *plantarum* FZB42(T)	CP000560	94.91

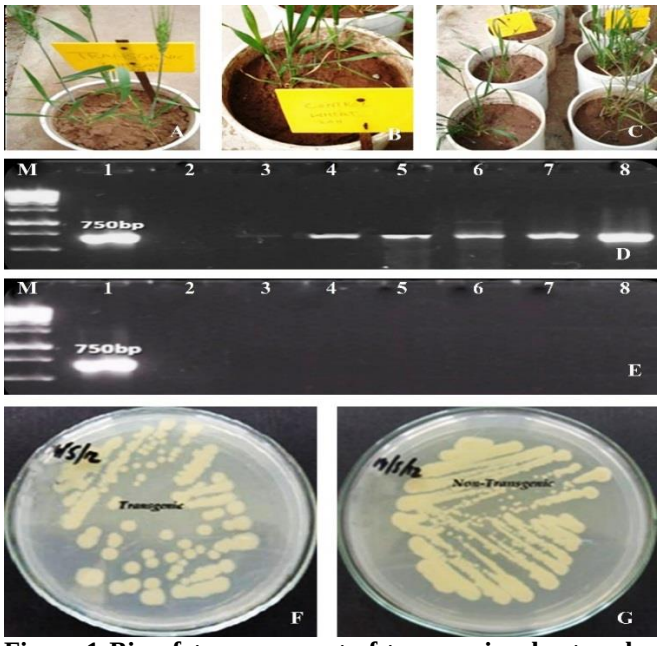

Figure 1. Biosafety assessment of transgenic wheat under greenhouse conditions (A, B): Transgenic wheat plants (C): Non-transgenic wheat plants (D): PCR analysis for the confirmation of *RCG3* gene in transgenic wheat plant where M shows I kb DNA ladder (Fermentas), Lane 1 shows positive control, Lane 1-2 shows non-transgenic wheat plants, Lane 4-8 shows transgenic wheat plants (E) PCR analysis for the confirmation of *RCG3* gene in wheat rhizosphere where Lane 1 shows positive control, Lane 2-4 shows control soil microflora, Lane 5-8 shows transgenic soil microflora (F) Bacterial colonies of transgenic wheat rhizosphere on LB medium (G) Bacterial colonies of non-transgenic wheat rhizosphere on LB medium.

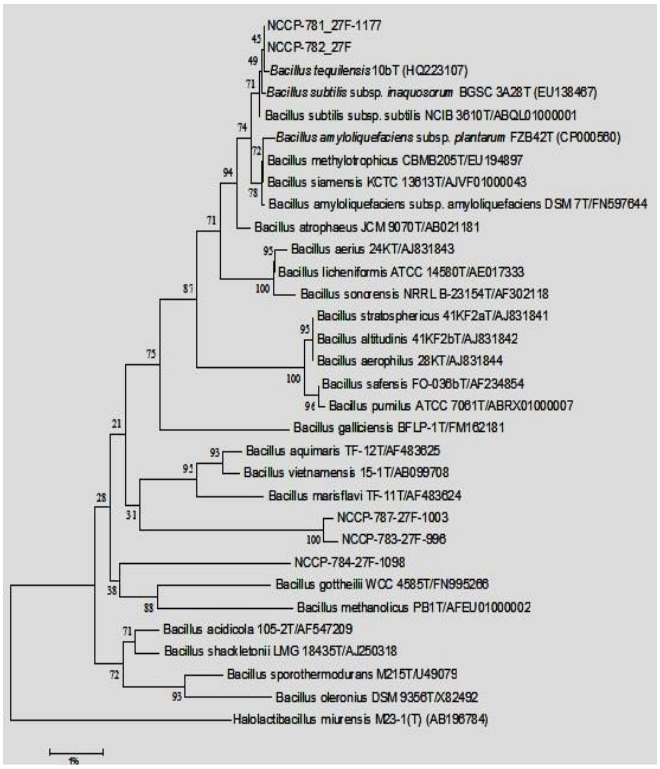

Figure 2. Phylogenetic tree of wheat rhizospheric soil bacteria.

Table 6. Assessment of resemblance of strains showing more than 94% similarities to NCCP 784 (Non-Transgenic) by phylogenetic analysis.

S. No	Related strains	Accession No.	Identity (%)
1	*Bacillus endophyticus* 2DT(T)	AF295302	97.95
2	*Bacillus shackletonii* LMG 18435(T)	AJ250318	94.88
3	*Bacillus circulans* ATCC 4513	AY724690	94.87
4	*Bacillus humi* LMG 22167(T)	AJ627210	94.62
5	*Bacillus subtilis*subsp*inaquosorum* BGSC 3A28(T)	EU138467	94.44
6	*Bacillus seohaeanensis* BH724(T)	AY667495	94.38
7	*Bacillus koreensis* BR030(T)	AY667496	94.35
8	*Bacillus smithii* NBRC 15311(T)	AB271749	94.17
9	*Bacillus subterraneus* DSM 13966(T)	FR733689	94.16

Similarly, NCCP-782 from control plant showed *Bacillus subtilis* subsp. *Inaquosorum* (97.95%), *Bacillus tequilensis* (95.34%), *Bacillus subtilis*(95.27%), *Brevibacterium halotoleran s*(95.2%), *Bacillus subtilis* subsp. *spizizenii* (95.2%), *Bacillus mojavensis* (95.12%), *Bacillus siamensis* (95.05%), *Bacillus vallismortis* (94.98%), *Bacillus myloliquefaciens* subsp. *Plantarum* (94.91%). NCCP 784 indicated more than 94% similarity with *Bacillus circulans* (94.87%), *Bacillus humi* (94.62%), *Bacillus subtilis* subsp. *Inaquosorum* (94.44%), *Bacillus endophyticus* (100),

Bacillus shackletonii (94.88%), *Bacillus seohaeanensis* (94.38%), *Bacillus koreensis* (94.35%), *Bacillus smithii* (94.17%) and *Bacillus subterraneus* (94.16%) (Tables 5 & 6; Fig. 2).

DISCUSSION

The knowledge of how an organism functions at molecular level had been started since 1970. An essential piece of this change has been the advancement of technologies that permit the gene transfer from one species to another. The speedy improvement of plant biotechnology has considerably promoted the growth of genetically modified crops (Lee *et al.*, 2006; Zhao, 2007; Basheer *et al*., 2016; Shahid *et al*., 2017). The worldwide cultivation of GM crops has developed enormous profit that may offer chances to solve the problems in world food safety but it has also created substantial biosafety anxiety (Bao, 2008). The probable hazard of GM organisms was formerly investigated by scientists in the early stages of the improvement of transformation technology. The risks linked with a GM crop relay on intricate contacts among the precise genetic alteration(s), the organism's normal history, and the distinctiveness of the ecological unit in which it is released. For this reason, transgenic crops must be tested for risk assessment prior to commercialization.

In the present study both vertical and horizontal gene flows were checked for transgenic wheat. The results showed that there was no vertical or horizontal gene flow from transgenic wheat to soil bacteria and control wheat plants. This study has provided positive information of biosafety concerns for transgenic wheat plants. Same sort of gene flow was also checked by Koga-Ban *et al*. (2004). They transformed rice chitinase gene in cucumber plant and then in next generations their gene flow was checked. They observed no rice chitinase gene flow from transformed cucumber plants. Jian-Rong *et al.* (2010) conducted research on transgenic wheat and proved that it had no harmful effects on environment. They stressed on the facts that it was necessary to carry out biosafety assurance of transgenic wheat before its release to market. Another proof of no gene flow in wheat was provided by Eastman and Sweet (2002). They mentioned that wheat had low risk in terms of gene flow from crop to crop and from crop to wild relatives. Our results were supported by the findings of Eastman and Sweet (2002). In the previous study, vertical gene flow from transgenic potato to non-transgenic potato was examined by Helen *et al.* (1994) in which they pointed out that no vertical gene flow had been practiced from transgenic to non-transgenic potato.

In present study, we also tested horizontal gene flow from wheat to soil bacteria and found that there was no gene flow in soil microflora. Wei *et al*. (2008) conducted research on transgenic rice to perceive the existence and activity of Cry protein in soil and its effect on soil microorganisms. This research group reported that transgenic rice expressing *Cry1Ab* gene had no computable undesirable cause on the key microbial rhizosphere soil. In a similar study of horizontal gene flow, Head *et al*. (2002) also concluded that *Bt* cotton had no gene flow to soil microflora.

In contrast to our findings, other researchers claimed that probable environmental risks in transgenic wheat allied with transgene escape through gene flow (both vertical and horizontal) were leading among biosafety concerns (Amand *et al*., 2000; Halfhill *et al*., 2001; Lavigne *et al.,* 2002). According to Chen *et al*. (2004) the gene flow incidence (1.1-2.2%) was recorded from cultivated rice (Minghui-63) to wild *O. rufipogon*. The same results of gene flow (2. 94%) from cultivated rice were also reported by Song *et al.* (2003a). The contradiction among our results and others might be due to the differences in form and amount of plant material, methodology and incubation conditions in laboratory. In addition to these differences, other factors such as protein nature, type of soil and soil microorganisms could influence the choice of decompose rates in different soils.

Our soil samples from both transgenic and control wheat were evaluated for nutritional analysis. The results of soil nutritional analysis revealed that the highest KNO_3 level (1.53 mg/kg) and maximum phosphorous contents (2.8 mg/kg) were recorded in transgenic soil sample of wheat. The results of soil pH showed that transgenic sample exhibited more basic pH 8.45 values, while maximum EC (0.38 dS/m) was also obtained in transgenic soil. Our results were slightly different from that of Tahira *et al.* (2011) who described that pH of wheat soil was 7.94 and EC was 4 dS/m. These differences might be due to variation in soil texture, heterogeneity in soil and environmental conditions from where soil was collected.

In our bacterial colony counting experiments, maximum colonies of 10^{-2} CFU/g dilution factor were obtained in transgenic wheat rhizosphere. The same results were also reported by Leeflang *et al.* (2002) who stated that *Pseudomonas putida* strain *WCS358r* both GMMs and control simultaneously decreased from 10^{-7} CFU/g of rhizosphere sample of wheat to 10^{-2}-10^{-4} CFU/g at harvest, and were in the range of detection border (10^{-2}-10^{-3} CFU/g rhizosphere sample) before harvesting. It has been revealed that no differences were observed when GMMs was exaggerated by the genetic alteration, as numbers of CFUs of the parental strain and the GMMs were in the same range. All the isolated bacterial strains were identified based on 16S rRNA gene sequencing. The same 16S rRNA gene was also used by (Ihara *et al.*, 1997; Stan lotter *et al*., 2002; Yoon *et al.*, 2001) for bacterial identification. The phylogenetic tree of our bacterial strains represented that all of them belonged to the genus *Bacillus* because members of the genus *Bacillus* were famous for their capability to emit many degradative enzymes such as chitinase (Schallmey *et al.*, 2004).

***Conclusion*:** Based on our biosafety tests, we concluded that no vertical gene flow was detected in control plants of wheat. Horizontal gene flow from transgenic wheat to soil microflora was also not detected in all experimental tests. There were no obvious differences in soil nutrients of transgenic and control plant soil. Outcomes of this study recommend that prior to commercialization; the transgenic wheat gene flow should be checked in isolated fields. Transgenic wheat should also be assessed under mixed cropping system. More detailed study including effects on animal regarding toxicity of inserted gene as well as nutritional changes in the seed must be evaluated. Metagenomic approaches for the identification of non-cultural bacteria should be applied to investigate the changes in the bacterial population.

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