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ALLELOPATHIC ASSESSMENT FOR THE ENVIRONMENTAL BIOSAFETY OF THE TRANSGENIC OILSEED RAPE LINES HARBORING THE ANTIFUNGAL SYNTHETIC CHITINASE (*NIC*) GENE

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Abstract

Brassica is famous for its contribution to fulfil the edible oil demand. Allelopathic tests were conducted on two varieties of *Brassica napus* i.e., Durr-e-NIFA (DN) and Abasyn-95 (AB-95) and their corresponding six transformed lines (DN-13, DN-120, DN-127, AB-11, AB-18, AB-31).The transgenic lines harbor synthetic chitinase gene (*NiC*) that confers resistance against *Alterneria brassicicola.* Allelopathic assessments were conducted on plant parts and the below ground soil samples of corresponding transgenic and non-transgenic lines using sandwich bioassay method. No difference was found in the effect of transgenic and non-transgenic lines (when using either fresh or dry leaf sample) on seed germination and seedling length of lettuce. Similar non-significant differences were found between the transgenic and non-transgenic lines for lettuce seeds germination in soil. The impact of root secreta from lines of both varieties was determined on fungi, actenomycetes and bacteria (microbes of the rhizosphere). Non-significant variation was found between transgenic and their corresponding non-transgenic lines for all the three tested microbes. In comparison to DN variety and the transgenic DN lines, the AB-95 variety and its transgenic lines showed slightly higher CFU (colony forming units) values. However, these differences were non-significant and considered to inherent differences between the two varieties. On overall basis, a substantial equivalence was considered on transgenic and non-transgenic lines for all the tested bioassays.

Key words: Allelopathy, Biosafety, *Brassica napus*, Sandwich bioassay, Soil microbes.

Introduction

Brassica napus is one of the most important oilseed crops and contributes 10% of the total edible oil consumption. With the rapid population growth and the resultant burden on food security, cultivation of oilseed rape crop has been focused as an important source of vegetable oil and a protein rich meal (Munir *et al.,* 2016). However, several environmental stresses including biotic stresses have negatively impacted the oilseed rape cultivation and yield (Dutta *et al.,* 2005). Biotic factors, particularly fungal pathogens cause a number of different diseases in oilseed rape crop resulting considerable economic losses throughout the world. Transgenic technology has been practiced for a long time to develop resistance in several crop plants including oilseed rape through transformation of antifungal chitinase genes isolated from various sources (Ahmad *et al.,* 2015; Gul *et al.,* 2015; Iqbal *et al.,* 2012). In this connection, the antifungal synthetic chitinase (NiC) gene was successfully transformed into *Brassica napus* plants and the expression of the transgene conferred stress resistance against *Alternariabrassicicola* (Khan *et al.,* 2013).

Apart from the introduced gene and the resultant trait, assessment of the negative impact of transgenic plants on environmental entities is an important biosafety consideration (Nickson, 2008). Parallel to this, assessment of the NiC gene and the chitinase protein on allelopathic potential and soil microorganisms is an essential regulatory requirement for the environmental safe use of transgenic *B. napus*. It is assumed and experimentally proved in some instances that transgenic plants may correspond or interact with the surrounding vegetation and soil microorganisms through production of allelochemicals, roots exudation and/or through fallen leaves and tillers. Soil microorganisms are essential components of the local ecosystem and have direct effects on plant growth and development (de Souza *et al.,* 2015). Moreover, chitin is one of the essential components found in the cell walls of many soil microorganisms. Therefore, transgenic plants harboring an antifungal chitinase transgene may directly or indirectly affect the soil microflora (Glandorf *et al.,* 1995).

Environmental risk assessment of transgenic plant is monitored in a sequential manner from highly controlled laboratory condition to semi-controlled greenhouse and finally in the confined field (Anon., 2002; Nickson, 2008). Allelopathic assessment of transgenic plants is considering an important factor of environmental risk studies (Kikuchi *et al.,* 2009). These assessments are important to investigate the effect of any unintended change of the transgenic plant due to transgene integration on the surrounding vegetation. Transgenic plants may produce allelochemicals, which may affect the growth, survival and spread of nearby plants, causing irreversible damage to biodiversity. For allelopathic effects on the surrounding plant vegetation, nature of the crop is investigated for production of any harmful compounds or allelochemicals. A number of research papers revealed that oilseed rape has allelopathic effect on a number of plants such as pasture, cereals and oilseed crops (Rice, 1984; Mason-Sedun *et al.,* 1986; Vera *et al.,* 1987; Anon., 2012). These allelopathic effects may include germination inhibition, reductions in root growth, plant height and seed yield (Anon., 2012). The allelopathic compounds are leached from the dead

plant parts such as leaves and stem by water on the upper soil layer. In this way, the oilseed rape may have competitive advantage through allelochemicals over the surrounding plant vegetation. This potential risk was previously evaluated as one of the key elements in environmental risk assessment studies on transgenic oilseed rape plants (Yoko *et al.,* 2011). Likewise, genetic modification with an antifungal gene may increase this competitive ability through allelochemicals, directly or indirectly and may pose a risk to biodiversity.

In the present study, the impact of the *NiC* transgene and its encoded chitinase protein will be assessed on the allelopathic potential between transgenic and non-transgenic lines of two *B. napus* varieties.

Material and Methods

The current study was conducted during 2015/2016 in Plant Genomics and Bioinformatics Laboratory of Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar.

Plant material and experimental design: Two varieties *of B. napus* (Durre-NIFA-DN) and Abaseen-95-AB-95) were genetically engineered with the antifungal synthetic chitinase (*NiC*) gene (Khan *et al.,* 2013). The transgenic lines were further evaluated for antifungal activity (Khan *et al.,* 2017). Three transgenic lines of Durre-NIFA (DN-13, DN-120, DN-127) and three transgenic lines of Abaseen-95 (AB-11, AB-18, AB-31) were used; whereas, the DN and AB-95 were used as the corresponding non-transgenic control plants. All the transgenic and non-transgenic lines were grown in small pots under growth room condition. The plants were then transferred to soil compost mix (50w/w) in large pots (5 Kg) and were kept under greenhouse condition. The pots were arranged in completely randomized design. For sandwich, soil germination tests, two trials were used; one before flowering and the other one after flowering stage. The microbial study was performed by a single trial on late vegetative phase of plants. Each experiment was replicated thrice and each replication consisted of 5 pots per line.

Semi-quantitative and quantitative real-time PCR analysis: This study was conducted to analyse the differential gene (transgene) expression in transgenic and non-transgenic plants. At the early vegetative stage, leaves were used for total RNA extraction using the RNeasy Plant Mini Kit (Thermo-scientific). An RNA sample of 5 µg was used for reverse transcription into cDNA through superscript II (Ivitrogen). Real-time PCR was conducted on ABI3700 based on the method described by Nakashima *et al*., (2007). Relative expression was conducted with the the*B. Napus Actin* gene mRNA. Primer sequences of *NiC* and *Actin* genes are given as below:

NiC forward 5′-GGTCGATGCCGTCCTCCTGTCCTT-3′
NiC reverse 5′-CGCCTTGGTGGTGGTCTTGATGGT-3′
Actin forward, 5′-TGAAGATCAAGGTGGTCGCA-3′
Actin reverse, 5′-AGAAGGCAGAAACACTTAGAAG-3′

Preparation of medium for allelopathic assessment: Sandwich bioassay was conducted on agar medium supplied with 0.75% (w/v) agar solution and 30-31ºC gelling temperature. The agar solution was autoclaved at 115ºC for 15 min and was then poured into six wells culture plates (costar 3516, USA). These plates were then used for the sandwich bioassay.

Sandwich method: Fresh leaf samples of 5 and 10 mg of all lines were taken in the culture plates according to the method described (Fujii *et al.,* 2003, Fujii *et al.,* 2004). A sample of 3 ml agar solution (0.75%) was poured on leaves tissues in each well. After solidification of the agar medium, extra 2 ml solution was added and then five lettuce seeds were placed in each well. Radicle, hypocotyls lengths and germination rate were measured after three days incubation of plates.

Fresh leaf samples of 5 and 10 mg of all lines were grinded in liquid nitrogen. Powder samples were placed in the culture plates for sandwich analysis as described by Fujii *et al.,* 2003, Fujii *et al.,* 2004). A sample of 3 ml of agar solution (0.75%) was added to each well on the powder samples. After solidification, another 2 ml agar solution was added to each well and five lettuce seeds were placed in each well. The germination rate, hypocotyl and radical lengths of lettuce plants were measured after three days of incubation.

Soil germination assay: Soil samples were collected from all potted plants of transgenic and non-transgenic lines. Lettuce seeds were grown on soil samples in each petri plate. The germination rate, hypocotyle and radicle lengths of lettuce seedlings were measured after incubation at room temperature in dark (Shiomi *et al.,* 1992).

Soil microbe analysis: Soil microbe analysis was conducted as described by Tabei *et al.,* (1994). Soil samples from transgenic and control lines were collected at post-flowering stage of plant growth. A sample of 30 g dry soil was added to 270 ml of 15 mM of sterilized phosphate buffer (pH 7.0). Further dilutions of the suspension were made from 102 to 105 with 15mMphosphate buffer. A 100 ml suspension was spread on the diluted PTYG agar plates and the bacteria and actinomycetes were detected as described by Balkwill and Ghiorse (1985). Fungi were detected by spreading a 100 ml suspension on the autoclaved rose bengal medium as described (King *et al.,* 1979). The rose Bengal medium consisted of 25 mg l^{-1} rose Bengal, 5 g l^{-1}Bacto-Peptone, 10 g l^{-1} glucose, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 g l^{-1} K_2HPO_4, pH 7.2, and 15 g l^{-1} agar. Fungal plates exposed with 25°C for 4 days before start counting of colonies.

Statistical analysis: One-way ANOVA was used to analyse the data of experiments (sandwich, soil germination and soil microbe). Means were compared using student t-test.

Results

Antifungal assay of the transgenic and wild type control lines: In our previous study, we conducted the antifungal analysis on similar NiC- lines of DN and AB-95 varieties (Khan *et al.,* 2017). Leaves extracted proteins of different lines were used to conducted the antifungal activity of against the mycellial growth of *Alternaria brassicicola* on potato dextrose agar (PDA) media. All transgenic lines showed antifungal activity expressed as % growth inhibition of fungal growth. Some of the transgenic lines such as AB-31 and DN-120 showed comparatively high antifungal activities.

Gene expression and quantification: Semi-quantitative and quantitative expression analysis of the *NiC*-transgenic plants was carried out to determine variable expression of the transgene (Figs. 1 and 2). All transgenic lines showed expression of the *NiC* gene. According to the expression analysis, DN-120 and AB-31 represent high NiC expression. However, no significant correlation was found between antifungal activity of *B. napus* (DN-120 and AB-31) and their relative *NiC*expression.

Effect of fresh leaves extract on germination, and root and hypocotyl length of lettuce plant: Effect of fresh leaves extracts of both transgenic and control lines were observed on lettuce seedling germination (Table 1). Data indicated that the leaves extracts have no significant ($p \leq 0.05$) effect on the germination rate of lettuce seeds.

The allelopathic data of fresh leaves of both transgenic and control plants showed no significant ($p \leq 0.05$) difference for all genotypes (Tables 1 and 2). Results indicated that maximum root length was recorded for DN-120 (15.3 mm). The results obtained for AB-95 variety also showed non-significant ($p \leq 0.05$) difference among transgenic and non-transgenic control lines where maximum root length was observed for AB-95 (13.5 mm) followed by AB-11 (13.4 mm) and AB-18 (13.4 mm). Least root length was observed for AB-31 (13.3 mm).

The allelopathic results showed no significant ($p \leq 0.05$) difference for all the transgenic and control lines of DN verity for the impact of fresh leaves on the hypocotyl length of lettuce seedlings (Tables 1 and 2). Maximum length was recorded for DN (14.9 mm) and DN-127 (14.9 mm), followed by similar results of DN-13 (14.8 mm) and DN-120 (14.8 mm). The results of data collected for AB-95 variety also revealed non-significant ($p \leq 0.05$) difference for all the transgenic and non-transgenic lines. Maximum hypocotyl length was recorded for AB-95 (13.9 mm) followed by AB-18 and AB-31 (13.8 mm), respectively. Least hypocotyl length was recorded for AB-11 (133.7 mm).

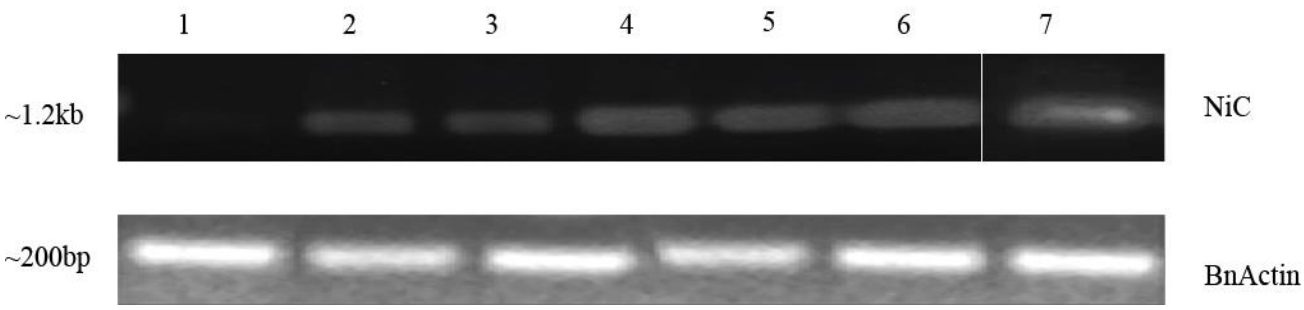

Fig. 1. Semi-quantitative PCR analysis of the *NiC* gene in the non-transgenic control and transgenic lines of both varieties. Line # 1 is the control line while lines # 2-7 are the transgenic lines. Actin mRNA was used as the internal control.

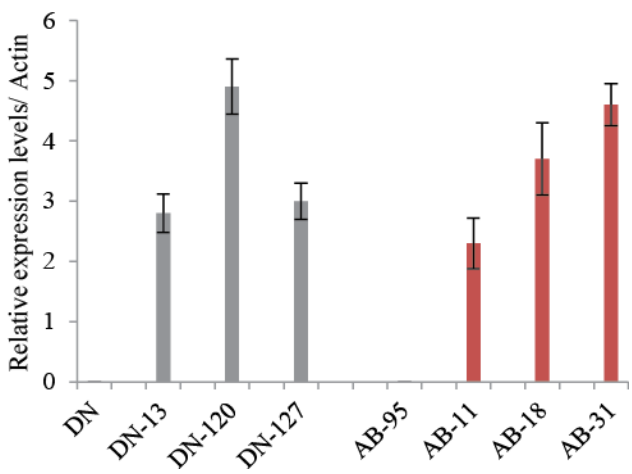

Fig. 2. Quantitative RT–PCR Analysis of the *NiC*-transgenic and wild type control plants of DN and AB-95 *B. napus*varieties. Transgenic lines of both varieties showed expression of the *NiC*gene, while no expression was observed in the control plants. The *B. napus Actin* gene mRNA was used as internal control.

Effect of dried and crushed leaves on seed germination, root and hypocotyl length of lettuce plant: The allelopathic effect of crushed leaves extract of transgenic and control lines were determined on the germination rate of lettuce seeds (Table 3). The data showed non-significant ($p \leq 0.05$) variation for all transgenic and control lines of the two varieties. All of the genotypes and lines which were sown on the agar media, supplemented with crushed leaves extracts were found with maximum germination rate.

The allelopathic effect of crushed leaves of both transgenic and non-transgenic genotypes on root length of lettuce seed is shown (Tables 3 and 4). The results indicated non-significant ($p \leq 0.05$) difference among the root lengths of lettuce seeds. Maximum root length for the DN variety was recorded for the non-transgenic DN line (13.8 mm) followed by DN-13, DN-120, and DN-127 (13.7 mm each). The outcomes of data recorded for AB-95 variety also revealed no significant ($p \leq 0.05$) variation between transgenic and the control lines.

The data recorded for the allelopathic effect of crushed leaves on hypocotyl length of lettuce seedlings exhibited no significant ($p \leq 0.05$) variation between transgenic and non-transgenic control lines (Tables 3 and 4). Maximum hypocotyl length was recorded for variety DN (14.0 mm), followed by an analogous pattern of 13.8 mm for DN-13, DN-120, and DN-127, respectively. Data recorded for AB-95 variety also showed no significant ($p \leq 0.05$) differences for all the transgenic and control lines, where maximum hypocotyl length was recorded for AB-95 (12.8 mm). On the basis of varietal differences, a significant variance can be seen in the data recorded for both varieties. The DN verity showed greater hypocotyl length (13.7 mm) as compared to AB-95 variety (12.7 mm).

Effect of soil on seed germination, root and hypocotyl length of lettuce: The lettuce seeds showed similar germination pattern in soil of *Brassica napus* lines irrespective of verity type and line is genetically modified or not (Table 5).

Data recorded for the root lengths of lettuce seedlings grown on soil sampled from transgenic and control lines showed no significant variation ($p \leq 0.05$) (Tables 5 and 6). Maximum root length for DN variety was showed by DN-127 (15.0 mm). Data recorded for variety AB-95 also showed non-significant difference ($p \leq 0.05$), where maximum root length was recorded for AB-95 and AB-31 (15.5 mm), respectively. On the overall basis, varietal differences were seen markedly i.e for DN variety, the value of root length was recorded to be 14.8 mm that is lower than that of AB-95 variety, where root length was recorded to be 15.4 mm implying effect of varieties rather than the genotypes.

Data recorded for the hypocotyl length of lettuce seedlings, grown on soil collected form transgenic and non-transgenic plant pots showed no significant variation ($p \leq 0.05$) (Tables 5 and 6). A similar sort of pattern was observed for the lines of DN variety, where maximum hypocotyl length was recorded for DN, DN-120 and DN-127 (16.7 mm), respectively. Least hypocotyl length was recorded for DN-13 (16.6 mm). For variety AB-95, non-significant differences ($p \leq 0.5$) were observed. Maximum hypocotyl length was observed for AB-95 and AB-11 (17.0 mm), followed by AB-18 (16.9 mm) and AB-31 (16.8 mm). On the overall basis, non-significant differences were observed between lines of the two varieties. The DN and AB-95 varieties showed 16.7 mm and 16.9 mm hypocotyl lengths, respectively.

Table 1. Effect of fresh leaves extract of transgenic and non-transgenic lines on rate of seed germination, and seedlings growth (root and hypocotyls lengths) of lettuce.

Lines	Germination rate (%)	Growth factor	
		Root length (mm)	Hypocotyl length (mm)
DN	99.7 ± 2.4	15.2 ± 2.1	14.9 ± 1.8
DN-13	98.9 ± 2.7	15.1 ± 1.9	14.8 ± 1.7
DN-120	98.6 ± 3.1	15.3 ± 2.1	14.8 ± 2.1
DN-127	99.1 ± 2.8	14.9 ± 1.8	14.9 ± 2.0
AB-95	98.7 ± 3.4	13.5 ± 1.5	13.9 ± 2.2
AB-11	98.5 ± 2.7	13.4 ± 1.4	13.7 ± 1.9
AB-18	99.1 ± 2.9	13.5 ± 2.4	13.8 ± 2.3
AB-31	99.3 ± 3.4	13.3 ± 2.3	13.8 ± 2.1

The data means the average ± SE of 6 samples

Table 2. *P*-values according to the pair-wise student *t*-test (at $p \leq 0.05$) of differences in lettuce seed growth affected by fresh leaves extract from transgenic and non-transgenic lines.

Varieties	Pair wise		Root length	Hypocotyl length
	Factor1	Factor 2		
DN	DN	DN-13	0.961	0.961
	DN	DN-120	0.975	0.946
	DN	DN-127	0.998	0.990
AB-95	AB-95	AB-11	0.994	0.991
	AB-95	AB-18	0.984	0.990
	AB-95	AB-31	0.997	0.996
Overall	DN	AB-95	0.984	0.995

Table 3. Effect of crushed leaves extract of transgenic and non-transgenic lines on rate of seed germination, and seedlings growth (root and hypocotyls lengths) of lettuce.

Lines	Germination rate (%)	Growth factor	
		Root length (mm)	Hypocotyl length (mm)
DN	99.4 ± 3.1	13.8 ± 2.0	14.0 ± 2.4
DN-13	97.9 ± 2.8	13.7 ± 2.2	13.8 ± 2.3
DN-120	98.2 ± 3.2	13.7 ± 1.7	13.8 ± 2.1
DN-127	98.1 ± 3.5	13.7 ± 2.0	13.8 ± 2.0
AB-95	98.2 ± 2.7	12.8 ± 1.6	12.8 ± 1.3
AB-11	97.5 ± 3.1	12.0 ± 1.7	12.7 ± 1.7
AB-18	97.6 ± 2.5	12.2 ± 2.1	12.6 ± 1.8
AB-31	97.3 ± 2.8	12.2 ± 2.2	12.7 ± 1.5

The data means the average ± SE of 6 samples

Table 4. Probability (*P*-value) according to pairwise student *t*-test (at $p \leq 0.05$) of differences in lettuce seed growth affected by crushed leaves extract from transgenic and non-transgenic lines.

Genotype	Pair-wise		Root length	Hypocotyl length
	Factor 1	Factor 2		
DN	DN	DN-13	0.962	0.955
	DN	DN-120	0.970	0.945
	DN	DN-127	0.976	0.996
AB-95	AB-95	AB-11	0.930	0.991
	AB-95	AB-18	0.943	0.986
	AB-95	AB-31	0.906	0.994
Overall	DN	AB-95	0.985	0.988

Table 5. Effect of soil from transgenic and non-transgenic lines on rate of seed germination, and seedlings growth (root and hypocotyls lengths) of lettuce.

Lines	Germination rate (%)	Growth factors	
		Root length (mm)	Hypocotyl length (mm)
DN	98.4 ± 2.4	14.9 ± 1.9	16.7 ± 2.5
DN-13	97.5 ± 2.3	14.8 ± 2.3	16.6 ± 2.3
DN-120	99.2 ± 3.4	14.9 ± 1.8	16.7 ± 2.2
DN-127	98.2 ± 3.3	15.0 ± 2.4	16.7 ± 2.4
AB-95	97.2 ± 4.1	15.5 ± 2.3	17.0 ± 2.5
AB-11	96.9 ± 3.4	15.4 ± 2.1	17.0 ± 2.4
AB-18	97.3 ± 2.8	15.3 ± 2.6	16.9 ± 2.1
AB-31	98.3 ± 2.7	15.5 ± 2.5	16.8 ± 2.2

The data means the average ± SE of 6 samples

Table 6. Probability (*P*-value) according to the pair-wise student *t*-test (at $p \leq 0.05$) of differences in soil effects on root and hypocotyl length between transgenic and non-transgenic lines.

Genotype	Pair-wise		Root length	Hypocotyl length
	Factor 1	Factor 2		
DN	DN	DN-13	0.986	0.995
	DN	DN-120	0.989	0.990
	DN	DN-127	0.997	0.997
AB-95	AB-95	AB-11	0.991	0.988
	AB-95	AB-18	0.980	0.989
	AB-95	AB-31	0.995	0.987
Overall	DN	AB-95	0.999	1.000

Table 7. The impact of root secreta on soil microorganisms (fungi, actinomycetes, and bacteria). Values are expressed as CFU g^{-1} dry weight of soil.

Lines	Fungus	Actinomycetes	Bacteria
DN	2.3×10^5	4.4×10^6	6.3×10^7
DN-13	1.9×10^5	4.8×10^6	6.1×10^7
DN-120	2.1×10^5	4.1×10^6	5.8×10^7
DN-127	2.2×10^5	4.2×10^6	5.9×10^7
AB-95	2.6×10^5	5.4×10^6	7.8×10^7
AB-11	2.4×10^5	5.3×10^6	8.1×10^7
AB-18	2.1×10^5	4.9×10^6	7.5×10^7
AB-31	2.2×10^5	5.5×10^6	7.3×10^7

CFU: The colony formation unit

Impact on soil microorganisms: The impact on soil microorganism is tabulated (Table 7). One-way ANOVA of the data revealed non-significant differences for fungi, actinomycetes and bacteria between the non-transgenic variety DN and transgenic DN-13 (P = 0.23, 0.89, 0.76, respectively), DN and DN-120 (P = 0.43, 0.79, 0.36, respectively) and DN and DN-127 (P= 0.89, 0.78, 0.64, respectively).All used lines ofAB-95 variety also showed the same trend. The One-way ANOVA showed non-significant differences for fungi, actinomycetes and bacteria between AB-95 and AB-11 (P= 0.78, 0.87, 0.67, respectively), AB-95 and AB-18 (P= 0.43, 0.72, 0.81, respectively), and AB-95 and AB-31 (P= 0.53, 0.84, 0.76 respectively). Collectively, the data indicated that microorganisms in the soil did not differ significantly among all transgenic and non-transgenic lines of the two varieties.

Discussion

Three different allelopathic tests were conducted to determine whether the *NiC* transgene in the transgenic *B. napus* lines has any negative effect on the surrounding plant vegetation and soil microorganisms. Allelopathic potential between the transgenic and non-transgenic control lines were determined through sandwich bioassay, soil germination method and soil microbe analysis. Data for all these tests showed no significant differences in transgenic and non-transgenic lines of DN and AB-95 varieties. Although, slightly variable expression levels were recorded for transgenic lines of both varieties and these differences were somehow reflected in the antifungal assay as previously described (Khan *et al.,* 2017). Previous reports on similar lines represent their inhibitory effect on causal fungi; however, no significant impact of these two lines was observed on the allelopathic parameters. It reveals that the constitutive expression of the synthetic chitinase gene has no allelopathic effect on the surrounding vegetation and soil microorganisms.

Similar allelopathic studies were previously conducted on several transgenic plants engineered with various biotic and abiotic stress tolerance traits. Yu *et al*., (2013a) conducted sandwich bioassay to determine the allelopathic potential of transgenic *Eucalyptus globulus*plants harboring the salt tolerance inducing *codA* gene. The study revealed no significant variation between the *codA*-transgenic and non-transgenic plants. In addition, results in the present study are in complete agreement with those of Shimazaki *et al*., (2009). They found no significant differences between the *Eucalyptus* non-transgenic and transgenic lines harboring the *Ntlim*gene. Further, Yu *et al*., (2013b) found non-significant differences between the non-transgenic and transgenic *Eucalyptus camaldulensis*plants harboring the *mangrin* transgene for salt stress tolerance. In our allelopathic results, small differences were found in the allelopathic effects of leaves collected before flowering and after flowering. This minor variation might be due to the reason that plants comparatively higher hypocotyl and root lengths of lettuce seeds were observed from plants before flowering stage as compared to those after flowering stage. The soil germination results showed that the transgenic and non-transgenic control lines of both varieties are not significantly different from each other in terms of germination rates of lettuce seeds. Our results are in complete agreement with those of Shiomi *et al.,* (1992). Similar results were reported for transgenic *Eucalyptus globulus* engineered with the *codA* gene (Yu *et al.,* 2013a). Transgenic *E. globulus* plants were not significantly different from non-transgenic control plants in terms of germination rates and sprout growth of lettuce seeds. In our results slight differences were noticed in the soil samples collected from plants before and after flowering stage. This variation might be due to the production of some metabolites in plants after the flowering stage. Shiomi *et al.*, (1992) used the same method for soil germination test.

In the current study, we found non-significant differences for the three tested soil microorganisms between transgenic and non-transgenic lines. Several studies reported the potential impact of microbial genes and the encoded proteins in transgenic plants on soil microorganisms. Some studies showed changes in the soil microbial community structure and enzyme activities. In one study, transgenic cotton plants exhibited negative effects on soil microbial activities (Chen *et al.,* 2012). Genetically engineered maize harboring the *Bt* gene had significant impact on the microflora of the rhizosphre (Castaldini *et al.,* 2005). In contrast, other studies showed no significant effect on soil microorganisms. The effect of xyloglucanase gene from *Aspergillusaculeatus*, engineered in popular for high cellulose content and elevated specific gravity was determined on soil fungi, actinomycetes and bacteria (Taniguchi *et al.,* 2008). Non-significant differences were found for these microorganisms between the transgenic and the non-transgenic control lines. Furthermore, non-significant effects were observed on soil microorganisms between non-transgenic and transgenic *B. napus* harboring glufosinate tolerance trait (Asanuma *et al.,* 2011). The *Bt* genes isolated from *Bacillus thuringiensis* conferring insect resistance have been extensively used in transgenic plants. Some of the transgenic plants were tested for the potential impact on soil microorganisms. Shinoyama *et al.,* (2008) evaluated environmental risk assessment studies on transgenic chrysanthemum transformed with a modified *cry1AB* gene. No significant effect of the transgene or the encoded protein was found on fungi, actinomycetes and bacteria in the soils of transgenic and control lines. The study further revealed that the number of tested microorganisms changed throughout the plant growth but were not significantly different between soils of transgenic and non-transgenic lines of both varieties of *B. napus.*

Conclusions

On overall basis, our results revealed non-significant differences for the tested allelopathic parameters between transgenic and non-transgenic lines of the two brassica varieties. Throughout the biosafety assessment, no substantial differences were found except expression of the chitinase gene and the resultant resistance to the target pathogen, *Alternariabrassicicola*. Findings of the present

study will add valuable information for assessing the potential allelopathic impact of antifungal chitinase and similar microbial genes in transgenic plants on the surrounding vegetation and soil microbial community structure. Moreover, such research findings will further strengthen the current efforts towards regulatory decision-making on the commercial release of transgenic oilseed rape crop.

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