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# PRODUCTION OF TRANSGENIC *BRASSICA JUNCEA* WITH THE SYNTHETIC CHITINASE GENE *(NIC)* CONFERRING RESISTANCE TO *ALTERNARIA BRASSICICOLA*

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**Abstract**

*Brassica juncea* is an important oil seed crop throughout the world. The demand and cultivation of oil seed crops has gained importance due to rapid increase in world population and industrialization. Fungal diseases pose a great threat to Brassica productivity worldwide. Absence of resistance genes against fungal infection within crossable germplasms of this crop necessitates deployment of genetic engineering approaches to produce transgenic plants with resistance against fungal infections. In the current study, hypocotyls and cotyledons of *Brassica juncea*, used as explants, were transformed with *Agrobacterium tumefacien* strain EHA101 harboring binary vector pEKB/NIC containing synthetic chitinase gene *(NIC)*, an antifungal gene under the control of cauliflower mosaic virus promoter (*CaMV35S*). Bar genes and *nptII* gene were used as selectable markers. Presence of chitinase gene in trangenic lines was confirmed by PCR and southern blotting analysis. Effect of the extracted proteins from non-transgenic and transgenic lines was observed on the growth of *Alternaria brassicicola*, a common disease causing pathogen in brassica crop. In comparison to non-transgenic control lines, the leaf tissue extracts of the transgenic lines showed considerable resistance and antifungal activity against *A. brassicicola*. The antifungal activity in transgenic lines was observed as corresponding to the transgene copy number.

**Key words:** Chitinase, Brassica, Transgenic plants, Antifungal activity.

## Introduction

*Brassica* is considered as avital source of vegetable oil and a protein-rich meal. It ranks third among the oil seed crops, following palm and soya oil. In addition, Brassica ranks fifth among economically important crops followed by rice, wheat, maize and cotton (Sidra et al., 2014; Tufail *et al.*, 2015).Various crop species in Brassica genus provide edible roots, leaves, stems, flowers and seeds. The four most widely cultivated species for oilseed and vegetable production in Brassica genus are *Brassica rapa*, *Brassica juncea*, *Brassica napus*, and *Brassica carinata*. Indian mustard [*Brassica juncea* (L.) Czern.] is one of the vital species in the genus *Brassica* (Rabbani *et al.*, 1998). One of the major constraints in productivity of this crop is damages due to *Alternaria* leaf spot, which is caused by *Alternaria brassicae* (Reis &Boiteux., 2010). It is the most threatening and widely distributed disease, which attacks oil seed rape, mustard and other *Brassicas* (Kumar, 1997). Plants are susceptible to this disease at all growth stages. In addition there are some other fungi which are important in a sense that they can cause seed borne infections like seed rotting, loss of germination, seed discoloration, seedling mortality, production of primary inoculum initiating early epidemics and production of mycotoxins that may cause illness to man and animals following ingestion (Frisvad & Samson., 1991).

Production of enzymes capable of degrading the cell walls of invading pathogenic fungi is a crucial component of the defense response in plants against fungal infections (Ceasar & Iqnacimuthu, 2012). Chitin, a linear b-1, 4-linked polymer of N-acetylglucosamine, is a major component of many fungal pathogens (Itoh *et al.*, 2003). Chitin is hydrolyzed by Chitinase enzyme, present in a variety of organisms such as bacteria, fungi, higher plants and animals (Itoh *et al.*, 2003). Recently, chitinases have been receiving attention because of their possible application for the biological control of chitin-as a major cellular component of pathogenic fungi.

In such threatening situation, plant transformation technique is a valuable tool for introducing resistant genes into plantswithout co-transfer of undesirable characteristics from foreign species. With the rapid advancement of recombinant DNA technology during the past years, a large number of expedient genes have been identified, isolated and modified to study their mode of function and regulation. *Agrobacterium*-mediated transformation of Brassica plants has been reported successful with *Brassica juncea* (Mathews *et al.*, 1990) in the area of hybrid seed production (Jagannath *et al.* 2002), herbicide resistance (Mehra *et al.*, 2000), oil quality (Das *et al.*, 2006), aphid resistance (Dutta *et al.*, 2005), salt tolerance (Zhang *et al.*, 2001) and phytoremediation (Zhu *et al.*, 1999).

With the help of genetic engineering, resistance in transgenic *Brassica* against fungal diseases can be conferred through constitutive expression of pathogenesis-related protein chitinases, which have important role in providing protection against pathogenic attacks (Frettinger *et al.*, 2006), stress responses (Kim *et al.*, 2003), and growth and development. Chitinases are glycosyl hydrolases catalyzing the degradation of chitin, an insoluble linear b-1, 4-linked polymer of N-acetylglucosamine. These are produced by a wide range of organisms including bacteria, fungi, insects, plants, and mammals and have been shown to play important physiological and ecological roles. Plants produce chitinases as a defense against chitin-containing fungal pathogens by inhibiting spore germination and germ-tube elongation and degrading hyphal tips (Collinge *et al.*, 1993).

In this study, we sought to introduce resistance to fungal pathogens in *Brassica juncea* by expressing an antifungal *NIC* gene through *Agrobacterium* mediated transformation using *Agrobacterium tumefaciens* strain

EHA101 (Hood *et al.*, 1986) harboring the binary vector plasmid pEKB. It was reported that purified chitinase inhibits fungal growth *in vitro* and that chitinases play a major role in defending plants against fungal infections (Khan *et al.*, 2008).

## Materials and Methods

All the experimental work was carried out at the Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture, Peshawar, Pakistan.

**Seed sterilization:** Seeds were washed with tap water, and surface-sterilized in sodium hypochlorite solution (1% active chlorine) with a drop of Tween 20 for 15–20 min, followed by rinsing 3-4 times with sterilized distilled water inside laminar flow cabinet.

**Agrobacterium *tumefaciens* strains and binary vector:** *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1986) harboring the binary vector plasmid pEKB/NIC was used to infect cotyledons and hypocotyls. The plasmid containing the synthetic *chitinase* gene was kindly provided by Dr. Ikuo Nakamura, Graduate School of Horticulture, Chiba University, Japan. The coding region of *NIC* (approximately 0.90 kb) was integrated between cauliflower mosaic virus 35S promoter and nopaline synthase terminator in a binary plasmid vector pEKH1 (Fig. 1).The construct, pEKB/NIC, harboring the synthetic *NIC* gene, encoding chitin-hydrolyzing enzyme, the gene for neomycin phosphotransferase II (*nptII*), which confers kanamycin resistance and the bar gene encoding the enzyme phosphinothricinacetyl transferase (PAT) which confers resistance to bialaphos, a nonselective herbicide.

*A. tumefaciens* was cultured overnight at 28ºC on a rotary shaker (130 rpm) in liquid lysogeny broth (LB) medium. A colony was picked from *Agrobacterium* stock culture with flattened end of toothpick and was placed in LB medium, (10 g $l^{-1}$tryptone, 5 $gl^{-1}$ yeast extract, 10 g $l^{-1}$NaCl, pH 7.2) containing 25 mg/l chloramphenicol, 50 mg/l kanamycin sulphate, and 100 mg/l spectinomycin under sterile conditions. The bacterial suspension was centrifuged for 20 min at 20°C at 8000rpm, and the supernatant was decanted and resuspended the bacterial pellet in Murashige and Skoog (MS) hormone-free medium, diluted to $A_{600}$ = 0.5. Inoculum prepared was used for transformation.

**Plant transformation:** *Brassica juncea* explants i.e., cotyledons and hypocotyls were infected with the *Agrobacterium* suspension (inoculum) for 8-10 min, blotted dry with sterilized filter paper to remove excess of bacteria and were then co-cultivated on 10 g $l^{-1}$ agar solidified modified MS (Murashige & Skoog, 1962) medium supplemented with 20 $gl^{-1}$ sucrose, different concentration of Benzylaminopurine (BAP) (1, 2, 3, 4) mg/l, and (0.1, 0.2, 0.3, 0.4,0.5) mg/l Naphthalene acetic acid (NAA) and 20 µM silver nitrate. Petri plates were covered with aluminum foil and were maintained under dark conditions in growth room for 3 days at 25±1◦C.

**Selection and regeneration of transgenic plants:** After co-cultivation, explants were washed with distilled water containing appropriate quantity of antibiotic (such as cefotaxime) after co-cultivation so that excess of bacteria was removed and infected explants were transferred to MS medium supplemented with 50 mg/l kanamycin sulphate (Km) as selective chemical and 200 mg/l cefotaxime as bactericide. The antibiotics were filter-sterilized and added after autoclaving of media. The cultured explants were maintained for a week and then were transferred to the MS containing 3 mg/l Benzylaminopurine (BAP), 0.3, 0.4 mg/l Naphthalene acetic acid (NAA), 1% agar (w/v), 100 mg/l Km, and 250 mg/l cefotaxime, 20µM silver nitrate with biweekly subculture to fresh medium. Approximately 2 months after co-cultivation, adventitious shoots 1–2 cm long were excised from the discs and cultured on phytohormone-free MS supplemented with 100 mg/l Km, 250 mg/l cefotaxime and 1% agar for rooting of putative transformants.

**PCR analysis**: Polymerase chain reactions (PCR) was employed to screen transformants for *NIC*, *bar*, and *nptII* genes integration. Genomic DNAs from kanamycin-resistant and control *Brassica juncea* callus were extracted using cetyltrimethylammonium bromide (CTAB) method. PCR was performed using genomic DNA as a target and the oligonucleotide primers (BexCo.Ltd, Japan) for *NIC*, *bar*, and *nptII* genes. The following primers were employed.

FP-NIC1, 5′-CGGGATCCGTCATGAGTCTGCTGGTCGC-3
RP-NIC2, 5′-ACGCGTCGACATCAGCAGCTCAGGTTCGGAC-3.

DNA template was denatured at 94°C for 4 min, then amplified by 30 cycles of amplification (30 sec at 94°C, 30sec at 62°C, 30 sec at 62°C and 1 min at 72°C), and a final extension was performed at 72°C for 5 min. The PCR product was expected to be 0.90 kb fragment for the *NIC* gene, 0.70 kb for the *bar* gene, and 0.80 kb for *nptII* gene. PCR products were separated by electrophoresis in 1% agarose gel.

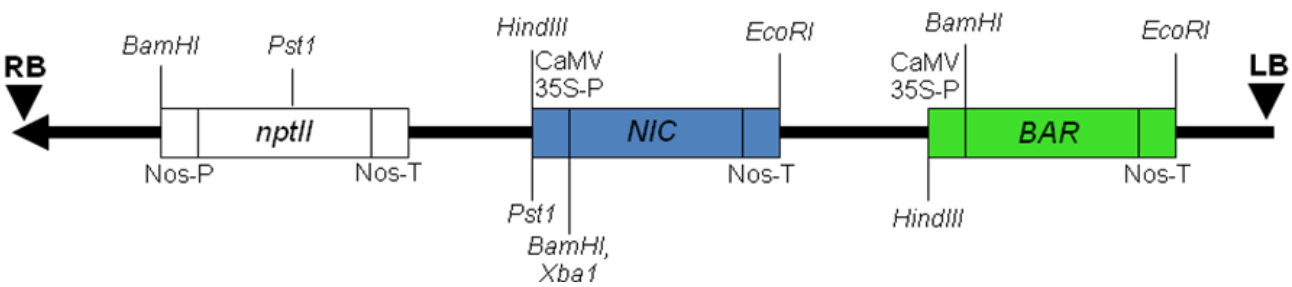


Fig. 1. T-DNA region of pEKH/NIC harboring chitinase and bar genes. The genes for chitinase and bar are driven by *CaMV 35S* promoter, and the gene for neomycin phosphotransferase (NPTII) by nopaline synthase promoter (nos-p). LB and RB, Right and Left border sequences of the T-DNA region, respectively.

**Southern blot hybridization:** Southern hybridization was conducted on DNA samples digested with *Hin*dIII enzyme. Digested DNA samples from kanamycin-resistant and non-transformed control plants were separated on a 0.8% (w/v) agarose gel. The gel was then blotted to a positively charged nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Amersham, UK) and hybridized with a digoxigenin (DIG)-labeled probe of the *NIC* gene. The probe DNA was labeled by PCR using DIG-dUTP (BoehringerIngelheim, Germany) and hybridization was conducted for 16 h at 41°C. Hybridization patterns were detected with the chemiluminescent substrate CDPStar (Roche Molecular Biochemicals, Mannheim, Germany) and anti-digoxigenin-AP (Roche Molecular Biochemicals, UK). The hybridized blot was exposed to X-ray film (Amersham Pharmacia Biotech) for 20 min at room temperature.

**Antifungal activity assay of extracted proteins:** For antifungal assay, proteins were extracted from leaf tissues. From the supernatant, a sample of 200 µl was taken and mixed with 2 µl of proteinase inhibitor. Then, the fresh suspension culture of *Alternaria brassicicola* was added to the extracted protein at a ratio of 1:9 and mixed. After through mixing, the mixture was spreadon potato dextrose agar (PDA) plates. The plates were kept for incubation at room temperature in the growth chamber with 16-h photoperiod. Inhibition potential of the extracts was then recorded in the –ve (control) and +ve plates. Also, the fungal colonies in CFU/cm$^2$ were recorded at 72 h of incubation (Ntui *et al.,* 2011). The experiment was conducted in two trials with three replications per trial.

**Statistical analysis:** The antifungal activity assay was set in a randomized complete block design with three replications. Analysis of variance (ANOVA) was used to analyze the antifungal activity data.

## Results

**Callus induction and transformation:** In this study, *Agrobacterium*-mediated transformation was employed to transform hypocotyls and cotyledons of *Brassica juncea.* Explants were infected with *Agrobacterium tumefaciens* strain EHA101 harboring the binary vector plasmid pEKB/NIC. *Bar* and *nptII* genes were used as selectable markers. Cotyledons and hopocotyls of 5-7 days old seedlings of *Brassica juncea* prior to transformation were pre-cultured on full strength MS media containing 2% sucrose, (1-4) mg/l BAP, (0.1-0.5)mg/l NAA, 1% Agar for 36 hours. In pre-cultured explants, rapid callus initiation was observed. Transgenic callus was efficiently produced when hypocotyls and cotyledons were used as transformation material. Explants when pre-cultured for 36 hours were more vulnerable to transformation and resulted in rapid transformed callus formation than those which were not pre-cultured as pre-culturing helps explants to get adapted to *in vitro* conditions.

Explants were infected with *Agrobacterium tumefaciens* strain EHA101 harboring the binary vector plasmid pEKB/NIC. After infection, hypocotyls and cotyledons of *Brassica juncea* seedlings were co-cultivated in full strength MS media containing 2% sucrose, (1-4) mg/l BAP, (0.1-0.5) mg/l NAA, 20 µM silver nitrate, 1% Agar at 25±1°C under dark conditions for 3 days. After keeping in dark for 3 days, explants were washed with liquid hormone-free MS containing appropriate quantity of antibiotic (such as cefotaxime), 100mg/l of cefotoxime helped in removal of excess of bacteria. Explants co-cultivated for 2-3 days were found to be sufficient for transformation of *Brassica juncea*. Temperature is believed to be an important factor to affect transformation rate. As temperature has impact on induction of *vir* genes which are responsible for transfer of T-DNA. When co-cultivation was carried out at 25±1ºC, most of the callus induced was transformed.

At all hormonal concentrations, callus initiation was observed. Bulging can be seen at the cut ends of explants. Callus initiations occurred nearly in first week after co-cultivation on all hormonal concentrations. Callus production was observed at all treatments and callus induction occurred at all hormonal concentrations. Calli were then transferred to MS supplemented with 50 mg/l kanamycin sulphate (Km) as selective chemical and 200 mg/l cefotaxime as bactericide. Efficient and rapid callus induction occurred at hormonal concentration containing 2 mg/l BAP and 0.2 mg/l NAA and 20 µM silver nitrate (Fig. 2A, 2B). In absence of silver nitrate, callus developed from few explants remained hard and compact. While in presence of silver nitrate, callus was large, soft and friable.

**Molecular analysis of DNA extracted from transgenic calli:** For PCR analysis, oligonucleotide primers specific to the *NIC* gene were used. PCR analysis amplified the 0.9 kb *NIC* gene in hypocotyls and cotyledons derived callus of *Brassica juncea* (Fig. 3A, lanes 1–4 and Fig. 3B, lanes 2,5,6,7 and 8) and were not detected in non-transformed callus (Fig. 3A, lane 5) and (Fig. 3B, lane 9).

The calli were maintained for 2 weeks and were then transferred to shoot regeneration media as described previously (Singh *et al*., 2009). The hormonal concentration of BAP (3 mg/l), NAA (0.5 mg/l), $AgNO_3$ (20 µM), agar (1% w/v), Km (100 mg/l) and cefotaxime (250 mg/l) showed good shoot induction (Fig. 4A, 4B).With this concentration, the addition of 20 µM $AgNO_3$ showed good results for regeneration. For rooting these transgenic shoots were transferred to MS medium without plant growth hormones after three months of infection as shown (Fig. 4C).

Genomic DNA was extracted from three transgenic and one non-transgenic control plants and was then subjected to PCR analysis to confirm the integration of the *NIC* gene in the plant genome. Shoots developed from transgenic callus also showed successful integration of *NIC* gene in the genome of these shoots as shown (Fig. 5A).

Southern blot analysis of the *Hin*d III-digested genomic DNA from transgenic plants showed the successful integration of either a single or multiple copies of the *NIC* gene (Fig. 5B). Independent lines designated as T1 and T7 showed two copies of the transgene (Fig. 5B, Lanes: 1 and 4), while T4 and T6 lines showed single copy integration of the transgene (Fig. 5B, Lanes: 2-3). No transgene insertion was detected in non-transformed control (Fig. 5, lane: 5).

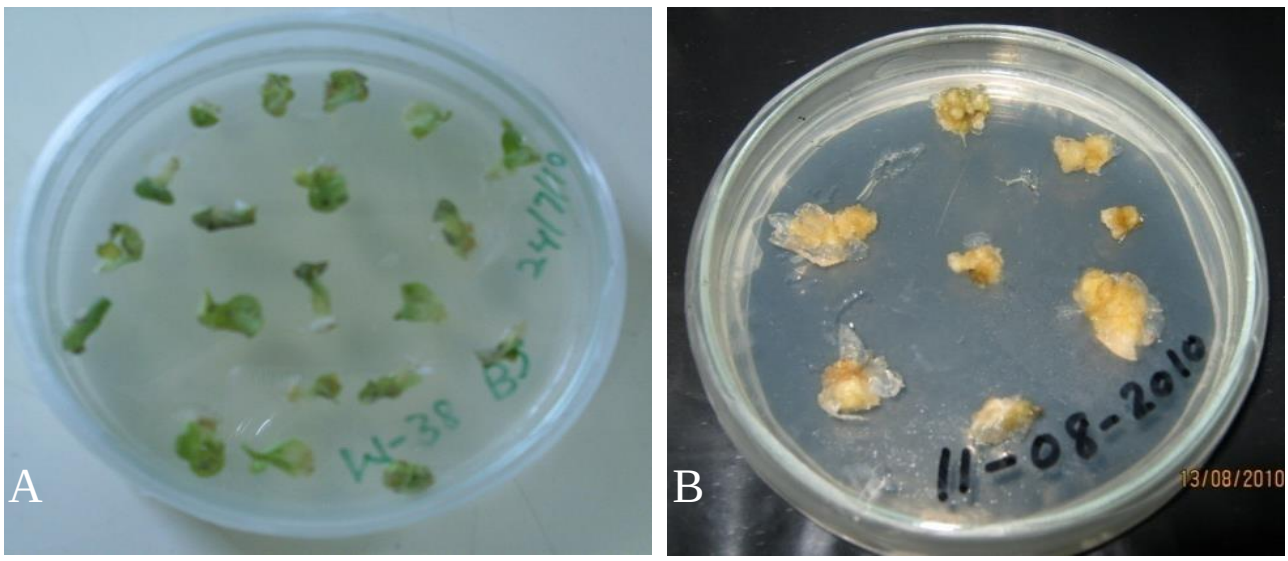


Fig. 2. Callus initiation in explants on callus induction media after co-cultivation (A). Transgenic calli on selection medium containing (BAP=2 mg/l and NAA 0.2 mg/l, 200 mg/l cef. Km 50 mg/l) (B).

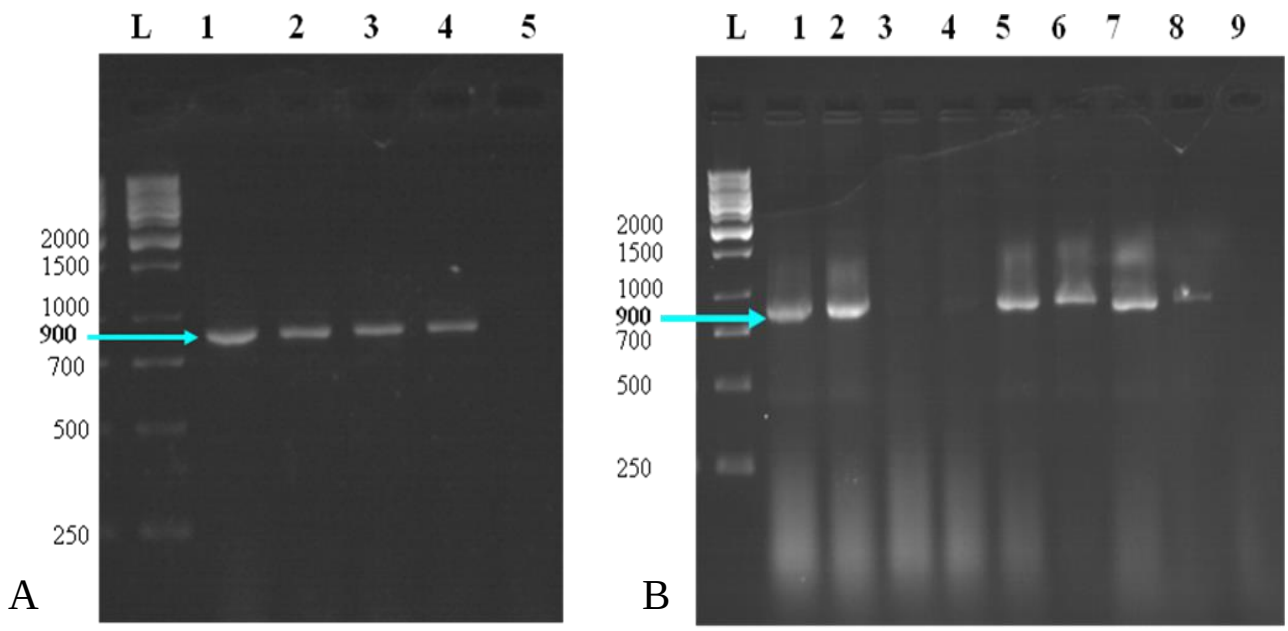


Fig. 3. PCR analysis of hypocotyls derived callus after infection with *A.tumefaciens* harboring binary plasmid vector, pEKB/NIC. L is 1 Kb ladder. Lane 1-4 is amplification of *NIC* gene (0.9 Kb) in transgenic callus**.** Lane 5 is Negative control (DNA from non-transformed callus) (A). PCR analysis of cotyledons derived callus after infection with *A. tumefaciens* harboring binary plasmid vector, pEKB/NIC. L is 1 Kb ladder. Lane 1 is positive control plasmid DNA for *NIC* gene (0.9 Kb). Lane 2-8 transgenic callus. Lane 2,5,6,7 and 8 is amplification of *NIC* gene (0.9 Kb) in transformed callus. Lane 9 is negative control (DNA from non-transformed callus) (B).

**Antifungal activities of leaf extract against *A. brassicicola*:** Antifungal activity assay was conducted and the effect of extracted proteins from leaf tissues was observed on the spore germination and growth of *A. brassicicola* at 72 hours post-incubation. After 72 h post-incubation, *A. brassicicola* showed significantly ($p \leq 0.05$) higher growth on control plates as compared to plates with proteins from transgenic lines (Fig. 6A). The non-transgenic control (NT) showed CFU/cm$^2$ value, 15.7 significantly ($p \leq 0.05$) higher than some of the transgenic lines. The transgenic lines T1, T4, T6 and T7 showed CFU/cm$^2$ values as 8.0, 11.3, 13.3, and 8.7, respectively (Fig. 6A). Further investigation was conducted to determine the inhibition potential of the extracted proteins from control and transgenic lines on the growth of *A. brassicicola*. On the control plates, no inhibition of the fungal growth was observed. On the contrary, some of the plates with proteins from transgenic lines showed considerable inhibition of the fungal growth (Fig. 6B). As was expected, the transgenic lines T1 and T7 that harbored double copies of the transgene showed maximum antifungal activity. Proteins from transgenic lines T1 and T7 showed approximately 15-20% inhibition potential as compared to control plates.

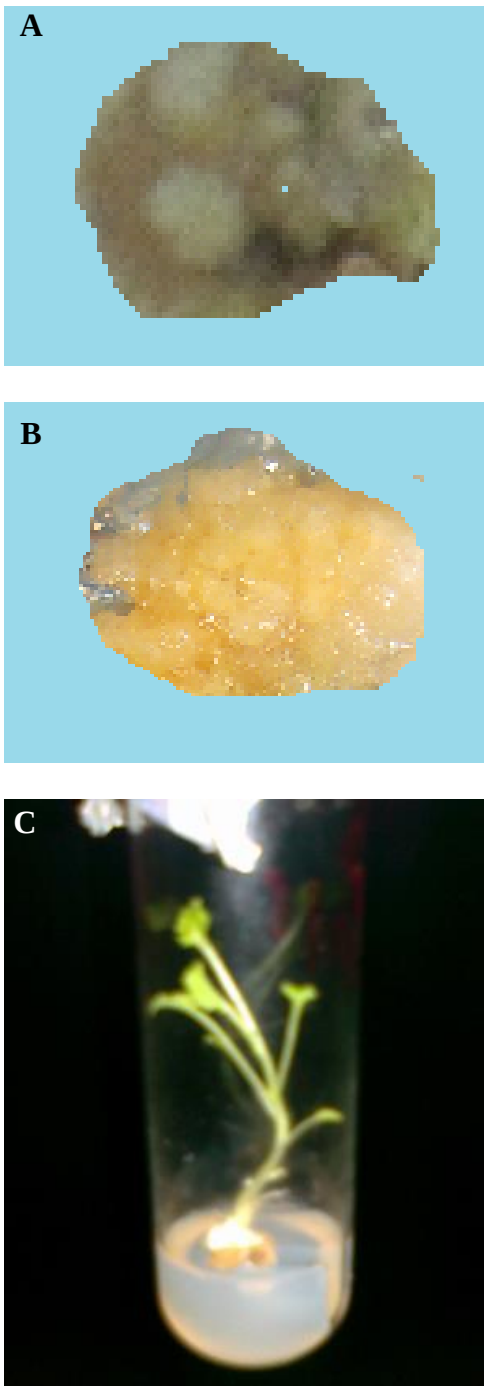


Fig. 4. Transformed callus on shooting medium with BAP 3 mg/l, NAA 0.5 mg/l, 100 mg/l Km and 250 mg/l cefotaxime is without silver nitrate (A), with 20 µM silver nitrate (B), and transgenic shoots and roots of *Brassica juncea* (C).

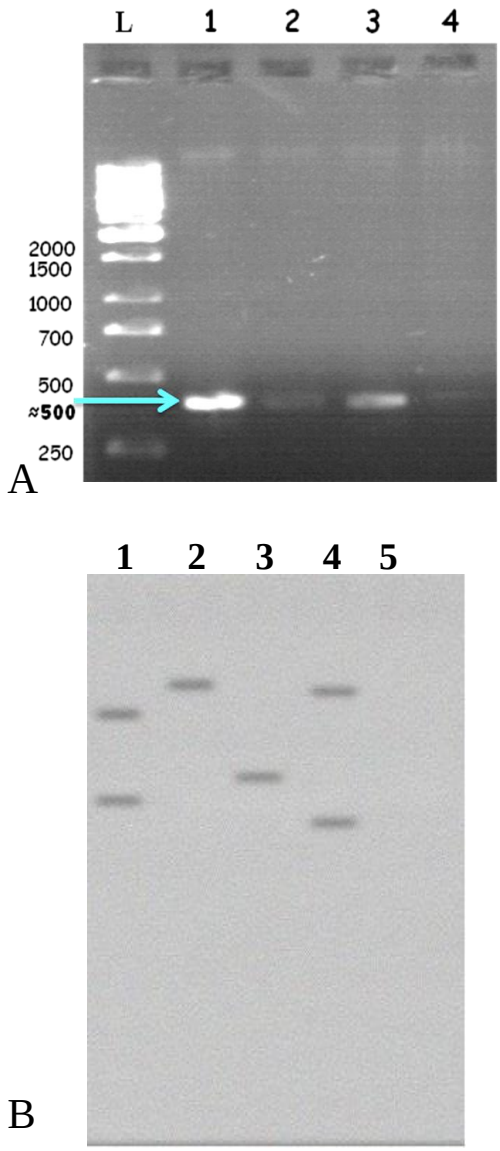


Fig. 5. PCR amplification of *NIC* gene from DNA of shoots. PCR Analysis of DNA extracted from 3 transformed and one non transformed control plants. L is the 1 Kb ladder, Lane 1-3, Independent explants samples in which *NIC* gene (~0.9 Kb) was amplified. Lane 4, DNA from control non-transformed plant, as a negative control (A). Southern blot hybridization of genomic DNA from transgenic and non-transgenic control *B. juncea* obtained after infection with *A. tumefacien* sharboring *NIC* and *nptII* genes. *Hin*dIII-digested genomic DNA from transgenic and non-transformed *B. juncea* was hybridized with a DIG-labeled probe of the *NIC* gene. Lane 1-4 (independent transgenic lines, T1, T4, T6, and T7), and lane 5: non-transformed control (B)

## Discussion

Chitinases have successfully been integrated into many plant species conferring resistance against different fungal pathogens (Ahmad et al., 2015; Gul *et al*., 2015). *Agrobacterium*-mediated transformation has been used to transform *Brassica* spp. Using standard tissue culture and non-tissue culture protocols (Naeem *et al*., 2016). In the current study, *Agrobacterium*-mediated transformation was employed to transform hypocotyls and cotyledons of *Brassica juncea*. In pre-cultured explants, rapid callus initiation was observed. Successful *Agrobacterium* mediated transformation is highly dependent on choice of explant used. It has been identified that transformation induced by *Agrobacterium* occurred mainly in cutide cells of explants (Longdou *et al*., 2005). To date, majority of transgenic *Brassica juncea* plants have been developed by using hypocotyl as explant and transformed with *Agrobacterium*-delivered vector systems (Dutta *et al*., 2005). In our study, we successfully transformed both hypocotyls and cotyledons of *B. Juncea*. Transgenic callus was efficiently produced when hypocotyls and

cotyledons were used as transformation material. It was observed that explants co-cultivated for 2-3 days were found to be sufficient for transformation of *Brassica juncea*. Longer co-cultivation period could result in necrosis of explants due to excessive growth of bacteria (Kim *et al.,* 2003). Our study also revealed the importance of temperature as an important factor affecting transformation rate. Thus 25±1ºC was the most appropriate temperature for exotic genes to insert and integrate into plant cell and result in effective transformation (Longdou *et al.,* 2005). Callus induction was observed at all hormonal concentrations; however, at 2mg/l BAP and 0.2 mg/l NAA, callus induction was significantly more efficient in *Brassica napus* (Kong *et al.,* 2009; Bano *et al.,* 2010). The presence of silver nitrate also positively affected callus production. The stimulatory effect of silver nitrate on transformation has been described (Delporte *et al.,* 2001). Hard and compact callus was observed in absence of silver nitrate with no significant increase in callus size. Thus, the silver nitrate presence was considered essential and 20µM was present in every step (Dutta *et al.,* 2008), in *Brassica napus* and *Brassica oleracea*. The hormonal concentration with the addition of Kanamycine and cefotaxime showed excellent shoot regeneration. Previously, it was observed that selection on media containing 50mg/l of Kanamycein and 200mg/l cefotaxime proved to be beneficial in inhibiting non transformed growth and in increasing incidence of transgenic callus production (De Block *et al.,* 1989) in *Brassica napus* and *Brassica oleracea*.

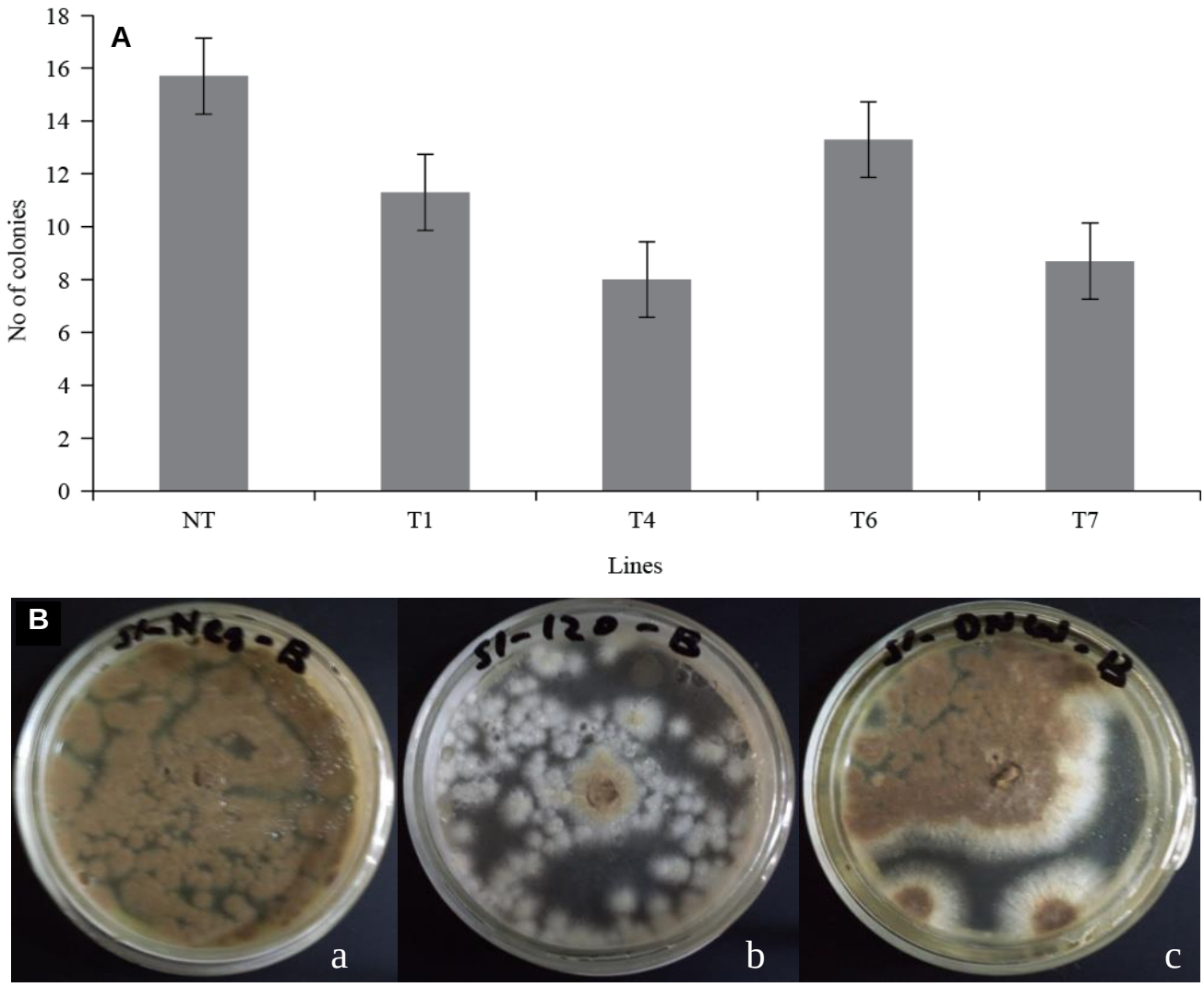


Fig. 6. Antifungal activities of transgenic *B. Juncea* plants expressing the *NIC* gene [Proteins were extracted from leaf tissues of transgenic and non-transgenic plants. Fungal colonies of *A. brassicicola* were counted at 72 hours post-incubation] (A). Fungal growth inhibition assay of transgenic and non-transgenic lines [Fungal growth inhibition of *A. brassicicola* was observed on plates containing protein extracts from non-transgenic plant (a), and transgenic lines (b and c) (B).

Antifungal activity of extracted proteins from leaf tissues was observed against *A. brassicicola*. In this study the transgenic lines with double copies of the transgene showed maximum antifungal activity. A number of previous studies have reported expression of the pathogenesis related (PR) genes such as chitinases and definsins in several plant species (Ntui *et al.,* 2010; Khan *et al.,* 2011; Ntui *et al.,* 2011; Hoshikawa *et al.,* 2012). In recent studies, Khan *et al*. (2012) reported resistance of transgenic Petunia with the synthetic chitinase gene (*NIC*)

to *Botrytis cinerea*. More recently, Kong *et al*. (2014) reported expression of the *NIC* gene in tobacco and tomato. The transgenic lines were observed to confer resistance to *Fusarium oxisporum*. In addition, some other studies described the expression of rice chitinase gene in transgenic grapevine, peanut and tomato plants that conferred fungal disease resistance (Nirala *et al.,* 2010; Iqbal *et al.,* 2012; Nyla *et al.,* 2015). Our study showed same results as those mentioned above.

## Conclusions

In the present study, transgenic *Brassica juncea* lines were developed which showed resistance to *A. brassicicola*, one of the major infecting pathogens of brassica species in Pakistan. Data in the present study suggests that the synthetic chitinase (*NIC*) gene as previously demonstrated in other plants is a suitable transgene for conferring broad-spectrum fungal resistance to *Brassica juncea*. The antifungal activity of transgenic lines was found correlated with copy number of the transgene.

## Acknowledgement

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