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AGROBACTERIUM-MEDIATED TRANSFORMATION OF *SPINACIA OLERACEA* L. THROUGH A SYNTHETIC CHITINASE GENE INDUCING RESISTANCE TO FUNGAL PATHOGENS

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Abstract

Agrobacterium-mediated transformation of spinach (*Spinacia oleracea* L.) Cv. Desi was carried out with a synthetic chitinase gene, *NIC.* The plasmid, *pEKH/NIC,* harboring the *NIC* gene, encoding chitin-hydrolyzing enzyme; the gene for neomycin phosphotransferase II (*nptII*), which confers kanamycin resistance and the *hpt* gene conferring resistance to hygromycin, was transformed into *Agrobacterium tumefaciens* strain EHA101. Hypocotyls and cotyledons from the 6-7 days old *in vitro* germinated seedlings and leaf discs from 3-4 weeks old green house grown plants of spinach were infected with overnight culture of the transformed *Agrobacterium.* Conditions such as co-cultivation period, bacterial concentration and concentration of plant growth regulators were optimized. Co-cultivation of explants with a bacterial concentration of1 x 10^9 cells/ml for 72 hours in dark, followed by culturing on a medium containing 20 $mgl^{-1}$and 200 mgl^{-1}cefotaxime resulted in an overall transformation efficiency of 23% for all the three explants. Transformation of spinach plants was confirmed by callus formation on selection medium, and PCR analysis using the primers for *NIC* gene. The results showed that T-DNA containing the *NIC* gene is integrated into genome of the spinach. Shoot regeneration, rooting, further molecular analysis and disease resistance tests of the transgenic plants are suggested to be carried out in future.

Key words: *Agrobacterium tumefaciens*, *NIC*, Synthetic chitinase, Transformation efficiency, Spinach.

Introduction

Spinach (*Spinacia oleracea* L.) is a leafy vegetable belonging to Chenopodiaceae family (Al-Khyari, 1995). Spinach is dioecious in nature, and is a diploid having a chromosome number 2n = 12. It requires long days, quite deep and extremely fertile soil for its development (Akhtar *et al.*, 2008). Amongst the world's healthiest vegetables, spinach is at the top of WHO's ranking list for its nutritional value. Rich in vitamins and minerals (Swiader *et al.*, 1992), it also contains health-promoting phytonutrients such as beta-carotene, lutein, xanthin and flavonoids offering remarkable protection against antioxidants. Because of its high nutritional value and antioxidant properties along with large amounts of vitamins present in it, spinach occupies a unique position among a variety of leafy vegetables.

As with most agricultural commodities, diseases lead to significant production losses equally affecting quantity and quality of *Spinacia oleracae* L. During its growing phase, spinach has to face several pests and diseases. Being a winter crop, it has to encounter fungal pathogens causing diseases including downy mildew, white rust, leaf spot, and seedling disease and fusarium decline. An integrated pest management approach, including the use of resistant cultivars, crop rotation, cautious irrigation, fertility management, and fungicides are sometimes necessary strategies to increase its yield (Correll *et al.*, 1994). Breeding is one of the strategies to develop spinach cultivars which are resistant to various pests and diseases, high temperature resistance, late flowering and having low concentrations of nitrate and oxalate. Although, a large number of cultivars have been developed using conventional breeding programs, it is still difficult to develop cultivars with desirable traits because of the lack of availability of germplasm. Genetic engineering is another idealistic strategy for successful integration of desired gene by using methods such as *Agrobacterium*-mediated transformation or particle bombardment. Thus, genetic engineering offers an array of opportunities in the improvement of this important vegetable crop.

Agrobacterium-mediated transformation is more efficient than other transformation strategies. The transgenic plants developed using *Agrobacterium*-mediated transformation is usually fertile and the alien genes are frequently transmitted to next generation (Rhodora & Thomas, 1996). *Agrobacterium tumefaciens* is extensively used for genetic transformation of plants because of its natural ability to transfer foreign DNA into the host plant genome. *Agrobacterium* mediated transformation protocols have been standardized in plants including eastern cottonwood (John *et al*., 2014), Mallee (Ahad *et al*., 2014), and many other plants.

The constitutive expression of one or more defense proteins in transgenic plants is required, to improve resistance against pathogens, using the techniques of genetic engineering. Amongst the defense molecules, chitinases occupy unique position because their activity against fungal pathogens has been reported in various crops. Chitin is an abundant β-1,4- linked homopolymer of *N*-acetyl glucosamine which is widely distributed in nature and is a structural component of fungal cell walls. Although plants lack endogenous chitin, they do express these chitinase enzymes which hydrolyze chitin. Chitinase is an enzyme which hydrolyses chitin at 1,4- ß-bonds between N-acetylglucosamine units. Chitinases are produced by a variety of organisms including bacteria (Lee *et al.*, 2007), fungi (Terakawa *et al.*, 1997), insects (Ding *et al.,* 1998), plants (Collinge *et al*., 1993; Kasprzewska, 2003), and mammals (Boot *et al*., 1995, 2005). Plants produce chitinases as a part of their defense

system against fungal pathogens containing chitin by inhibiting spore germination and germ-tube elongation and degrading hyphal tips (Collinge *et al.*, 1993; Graham & Sticklen, 1993).

So far, there have been few reports on genetic transformation of spinach which include, *Agrobacterium*-mediated transformation and regeneration of root explants of spinach (Knoll *et al*., 1997), *Agrobacterium*-mediated transformation of spinach leaf discs with cucumber mosaic virus (CMV) and confirmation of CMV coat proteins in transgenic plants (Yang *et al.*, 1997), over-expression of the green fluorescence protein (GFP) gene in spinach cotyledons after co-cultivation with *Agrobacterium tumefaciens* (Zhang & Zeevart, 1999), *Agrobacterium rhizogenes* has also been reported to be used for spinach transformation (Ishizaki *et al*., 2002). The transformation and regeneration efficiencies in spinach are highly affected by cultivar and types of explants used for infection with *Agrobacterium*, giving a range of 0–87% for transformation and 0–96% for regeneration efficiencies. For further improvement of the transformation and regeneration ability of large numbers of spinach cultivars an *Agrobacterium tumefaciens*-mediated transformation system have been developed which involves the use of an efficient plant regeneration system with a specific temperature treatment and use of hygromycin (kanamycin) for selection (Chin *et al.,* 2009).

Chitinases have successfully been integrated into many plant species including carrot (Punja & Raharjo, 1996), tobacco (Broglie *et al*., 1991), strawberry (Asao *et al*., 1997), peanut (Rohini & Rao, 2001), cotton (Tohidfar *et al.*, 2005), lemon (Gentile *et al*., 2007), tomato (Tabaeizadeh *et al*., 1999), rice (Dutta *et al*., 2001), wheat (Bliffeld *et al.,* 1998), brassica (Ahmad *et al.,* 2015) and potato (Khan *et al.,* 2008) to confer resistance against different fungal pathogens. In present research, an attempt has been made to transform spinach plants with a synthetic chitinase i-e *NIC* (Nakamura Ikuo Chitinase) gene encoding a broad spectrum chitinase protein, to enhance resistance against fungal pathogens. *NIC* gene has a size of 1271-bp, sharing 82% sequence homology with chitinase1 gene (*chi1*) from a zygomycete filamentous fungus, *Rhizopus oligosporous* (RCHCHITI, Yanai *et al.*, 1992). Hypocotyls, cotyledons and leaf discs were used as explants for transformation of spinach with *NIC* gene conferring resistance against fungal pathogens.

Materials and Methods

Plant material: Seeds of spinach cultivar "Desi" (kindly provided by National Agriculture Research Center, Islamabad, Pakistan) were used for transformation. Seeds were first dipped in 70% ethanol for 1 minute. Then Surface-sterilized in 4% sodium hypochlorite solution with a few drops of Tween 20 for 10 minutes. Finally rinsed 3- 4 times with sterilized distilled water inside laminar flow cabinet. Seeds were germinated on half strength Murashige & Skoog (1962) medium with 2% (wt/vol.) sucrose and 0.8% (wt/vol.) agar in flasks and incubated at 20 ± 5°C. Hypocotyls and cotyledons from 6-7 days old in vitro grown plants were excised and used as explants. Leaf explants excised from 3-4 weeks old green house grown plants were dipped in 70% ethanol for 30 seconds, followed by surface sterilization with 0.78% sodium hypochlorite for 5 minutes. Leaves were finally washed 3-4 times with sterile water. Leaf explants were pre-cultured on MS medium containing 0.5 mg l^{-1}2,4-D and 2 mgl^{-1}kinetin, while for hypocotyls and cotyledons an MS medium containing 2.5 mgl^{-1} BAP and 3mgl^{-1} 2,4-D, and 2.0 mgl^{-1} BAP and 2.5 mgl^{-1} 2,4-D was used respectively, for 72 hours.

Bacterial strain and plasmid: *Agrobacterium tumefaciens* strain EHA101 harboring binary vector plasmid *pEKH* consisting of 1.2 kb of DNA fragment encoding the *NIC* gene that was artificially synthesized and same as that of chitinase 1 gene (Chi1) in *Rhizopus oligosporus,* a zygomycete filamentous fungus (Yanai *et al*., 1992) and show 82% homology with the Chi1 gene was kindly provided by Dr. Ikuo Nakamura, Graduate School of Horticulture, Chiba University, Japan. The genes for *NIC* and hygromycinphosphotransferase (*hpt*) are driven by cauliflower mosaic virus 35S promoter (35SP), and the gene for neomycin phosphotransferase (*nptII*) by nopaline synthase promoter (nos-p). The terminators (T) of the *NIC*, *hpt* and *nptII* genes are derived from the nopaline synthase (nos). *pEKH*binary vector plasmid harbors hygromycin resistant (*hpt*) gene, and neomycin phosphotransferase (*nptII*) gene which confers kanamycin resistance (Bevan, 1984) (Fig. 1).

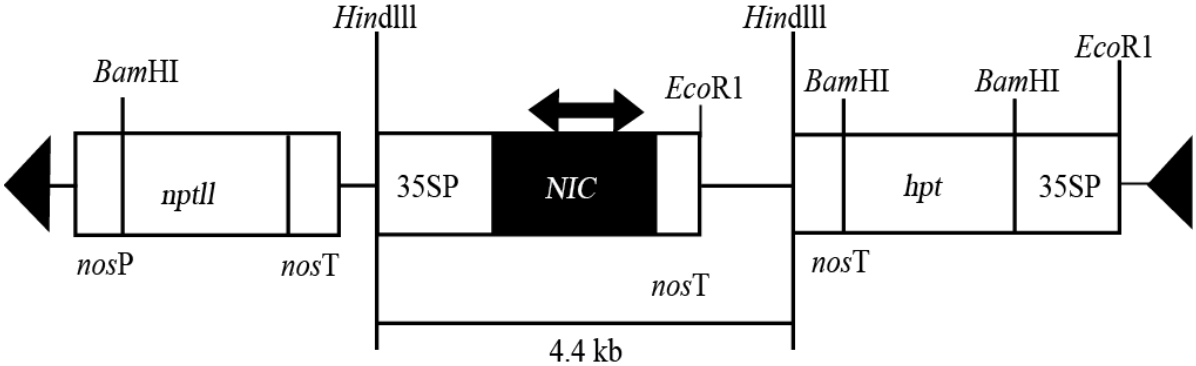

Fig. 1. T-DNA region of the binary vector pEKH used for spinach transformation. The NIC gene is driven by CaMV 35S promoter, and the gene for neomycin phosphotransferase (NPTII) by nopaline synthase promoter (nos-p). LB and RB, Right and Left border sequences of the T-DNA region, respectively.CaMV 35S-P cauliflower mosaic virus 35S promoter, nos-T terminator of the nopaline synthase gene. Recognition sites of restriction enzymes (*Bam*HI, *Hin*dIII, *Eco*RI and *Xba*1) are also indicated.

Preparation of *Agrobacterium* inoculum for infection: *Agrobacterium* was cultured overnight in 30ml LB liquid medium (10g l^{-1}tryptone, 5g l^{-1} yeast extract, 10g l^{-1}NaCl, pH 7.2), containing 25mgl^{-1}chloromphenicol + 50mgl^{-1} Km (kanamycin sulphate) in 100 ml flask, and placed overnight for growth on shaking incubator at 28°C. Then the optical density (OD_{600}) of the overnight culture was observed using spectrophotometer, which appeared to be 0.9. Then about 15 ml from the overnight culture was taken, and centrifuged in two 15 ml falcon tubes at room temperature, 8000 rpm for 10 minutes. Then the supernatant was discarded and the pellets were dissolved in about 1 ml of the supernatant in each tube and put in pre-autoclaved 50 ml MS liquid medium, in two 100 ml flasks to prepare inoculums for infection. Explants were infected by dipping 10-15 minutes in the inoculum prepared from *Agrobacterium tumefaciens* strain *EHA101* having the binary vector plasmid *pEKH/NIC* in flasks.

Infection and Co-cultivation: Explants were infected by dipping 10-15 minutes in the inoculum prepared from *Agrobacterium tumefaciens* strain*EHA101* having the binary vector plasmid *pEKH/NIC* in flasks. Explants were blotted dry on a sterile tissue paper after infection, and co-cultivated on plates containing 30ml MS medium (agar-solidified), having different combinations of growth regulators(2,4-D, Kinetin, and BAP). Controls were treated in the same way but without *Agrobacterium* infection. Then all the co-cultivated samples and controls were wrapped completely in aluminum foil and placed in dark room maintained at 20 ± 5°C.

Sub-culturing and selection: After co-cultivation for 3 days, all the infected explants and controls were transferred to selection MS medium containing 20 mgl^{-1} kanamycin with 200 mg l^{-1} cefotaxime with an interval of 3-4 days and finally at300 mg l^{-1} cefotaxime for 2-3 weeks. Transgenic calli produced after 6 weeks, were then subjected to PCR analysis. DNA was extracted from a small piece of transformed calli using CTAB method.

PCR analysis: Successful integration of *NIC* gene in transformed calli was confirmed using PCR analysis. Genomic DNA from kanamycin-resistant calli and non-transformed spinach plants was extracted using cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1988). PCR was performed using genomic DNA as a target and the oligonucleotide primers for *NIC* gene.

Shoot regeneration: Transformed calli were then transferred to regeneration medium, which was agar-solidified MS medium containing 0.01mgl^{-1} 2,4-D, 1mgl^{-1} kinetin, 1.0 mgl^{-1} GA_3, 10.0 mgl^{-1}meropenemtrihydrate and 100mgl^{-1} kanamycin, and incubated at 14°C.

Results and Discussion

Plant material and explants: *Agrobacterium*-mediated transformation of 3 different types of explants i-e hypocotyls, cotyledons and leaf segments, was carried out for successful integration of *NIC* gene. Spinach seeds were first surface sterilized, and then cultured on half strength MS medium. Seeds started germination after 2 days. Hypocotyl and cotyledon explants were excised from *in vitro* grown spinach plants and were pre-cultured on hormone supplemented MS medium, containing different combinations of 2,4-D and BAP.

Effect of plant growth regulators: In case of cotyledon explants callus induction was most efficient on the medium containing 2.0 mg l^{-1} BAP and 2.5mg l^{-1} 2,4-D, while callus producing response of hypocotyls was best on MS medium containing2.5 mg l^{-1} BAP and 3 mg l^{-1} 2,4-D, leaf explants efficiently produced calli on MS medium containing 0.5 mg l^{-1} 2,4-D, 2 mgl^{-1} kinetin was most efficient on the medium containing 0.5mg l^{-1} 2,4-D and 2mg l^{-1} while in case of hypocotyls and cotyledons, medium containing 2.5mgl^{-1} BAP and 3mgl^{-1} 2,4-D. The explants from green house grown plants were excised by cutting the leaves from the edges, and using the middle portion for transformation which was cut into small pieces because transformation efficiency has a direct relationship with wounded area i-e greater the wounded area more efficient will be the transformation.

Confirmation of transformed *Agrobacterium* with PCR analysis: *Agrobacterium tumefaciens* strain *EHA101* was transformed with binary vector plasmid *pEKH-NIC* harboring *NIC* gene. After transformation the *Agrobacterium* cells were grown on agar-solidified LB medium containing 50mgl^{-1} kanamycin and 25mgl^{-1} chloramphenicol and were incubated at 28°C. Colonies were observed after 24 hours. The plasmid from the *Agrobacterium* colonies was extracted using alkaline lysis method, and was confirmed using PCR analysis. PCR analysis of transformed plasmid revealed following results in 2% agarose gel (Fig. 2).

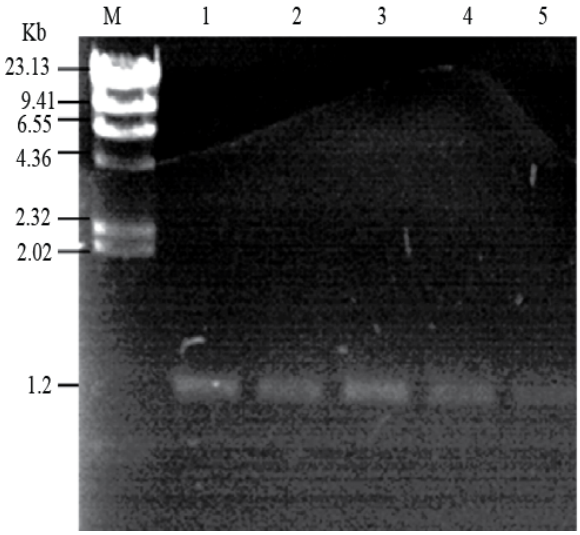

Fig. 2. PCR Analysis of *Agrobacterium* plasmid DNA. PCR analysis of plasmid DNA extracted from the colonies of *Agrobacterium tumefaciens* grown on LB medium using the *NIC* gene primer. M, size marker (*øX174/HaeIII*digest). Lane 1-5, Independent colonies in which *NIC* gene (1.2 Kb) was amplified.

After confirmation of *A. tumefaciens* transformation, glycerol stock of the *Agrobacterium* harboring the *pEKH-NIC*, was prepared, by picking single colony and mixing it with liquid LB medium and glycerol (20%), and was stored at -80^{O}C.

Explants infection in bacterial inoculums: The explants were dipped in *Agrobacterium* inoculums (OD=0.4-1.0), for 10-15 minutes, to carry out transformation. An optical density of 0.9 was proved to be more efficient for successful transformation of explants. Then the explants were blotted dry on sterile tissue paper, and transferred to Petri dishes, having MS medium inside laminar flow cabinet. After covering the petri dishes with aluminum foil, the infected explants were incubated in dark at a temperature of 20 ± 5^{O}C.

Callus initiation after co-cultivation: Few days after co-cultivation the explants started callus initiation at the wounded portion of the explants.

Formation of transformed calli: Transformed calli were obtained 6 weeks after co-cultivation, on selection medium containing suitable combination of growth regulators for different explants along with Kanamycin 20 mg l^{-1} and cefotaxime 200 mgl^{-1} .Calli were formed from leaf explants on selection medium containing 0.5 mg l^{-1} 2,4-D, 2 mg l^{-1} kinetin, 20 mg l^{-1} kanamycin and 200 mg l^{-1} chloramphenicol, 6 weeks after co-cultivation.

PCR confirmation of transgenic callus: Genomic DNA was extracted from transgenic calli developed on MS medium containing the antibiotic as a selective chemical and was subjected to PCR analysis using the primers for the target gene, *NIC* with a positive sample containing plasmid DNA. Then the PCR product was run on 2% agarose gel with a size marker of 1.2kb. The electrophoreogram indicating transformed calli is shown in Fig. 3.

Transformation efficiency: Almost 15% of the explants which did not responded well to pre-culture medium were excluded while calculating transformation efficiency (Table 1).

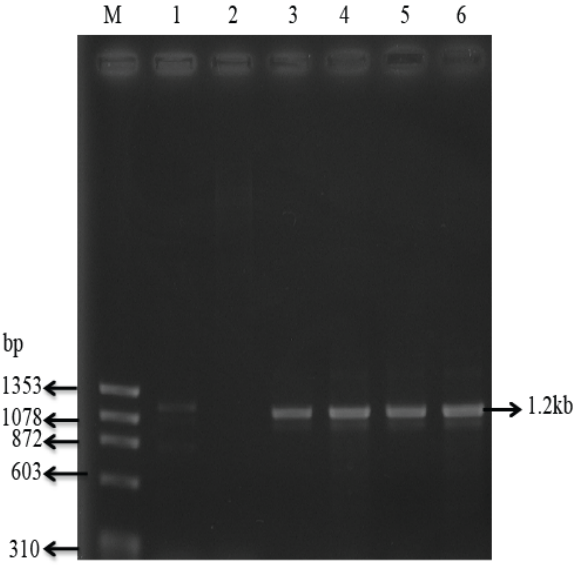

Fig. 3.Confirmationof *pEKH-NIC* in transformed shoots through PCR. M: size marker (bp) (*øX174/Hae*III digests).Lane 1: positive control, Lane 2: negative control, Lanes 3-6: genomic DNA from transformed plants

Table 1. Transformation Efficiency of different explants of Spinach.

Type of explants	Total number of explants used	Number of explants damaged	Number of transformed calli
Leaf discs	60	19	17
Hypocotyls	60	15	8
Cotyledons	60	13	11

Shoot regeneration: After confirmation, transformed calli were transferred to shooting medium containing 0.01 mgl^{-1} 2,4-D, 1 mgl^{-1} kinetin and 1 mgl^{-1} GA_3 . No shoots were developed due to un-availability of required temperature conditions.

The transformation and regeneration efficiency of spinach depends upon many factors such as the cultivar, explants types, temperature, plant growth regulators, photoperiod, light intensity, and *Agrobacterium* strain (Yang *et al*., 1997; Zhang & Zeevart, 1999; Geekiyanage *et al.*, 2006; Bao *et al*., 2009; Chin *et al*., 2009; Shojaei *et al*., 2010). Leaf segments, hypocotyls and cotyledon explants were used for transformation as reported previously (Yang *et al*., 1997; Zhang & Zeevart, 1999; Bao *et al.*, 2009; Chin *et al.*, 2009). After 72 hours of pre-culture, more than 80% of the explants increased in size because of the cell division. Only those plants, which responded well to pre-culture medium, were selected for transformation.

Total number of explants used was 180, many explants were damaged, and average transformation efficiency of all the three explants was found to be 23%. Pre-culture of the explants proved to have significant impacts on transformation efficiency. The effects of optical density and co-cultivation period were also studied, and results revealed that $0D_{600=}$ 0.9 and a co-cultivation period of 72 hours lead to maximum transformation efficiency (23%). Other published protocols for *Agrobacterium*-mediated transformation of spinach described co-cultivation of explants with several different strains of *Agrobacterium* (EH101 / pIGI21-Hm, LBA4404 / pBI121, EHA105 / pBE2111FMB, and LBA4404)), with OD_{600}= 0.4-1.0(Yang *et al.*, 1997; Zhang & Zeevart, 1999; Bao *et al*., 2009; Chin *et al.*, 2009) from 24-72 hours. When the optical density exceeded 0.9, and co-cultivation time was more than 72 hours, it had negative impacts on health as well as transformation efficiency of explants. It was noted that

explants which were not dried, after infection lead to overgrowth of *Agrobacterium*, which consequently had negative impacts on health of explants. That is why, it is vital to remove surplus bacterial deferment prior to co-cultivation.

Plant growth regulators indicated significant impacts on regeneration. For leaf explants MS medium containing $0.5mgl^{-1}$ 2,4-D and $2mgl^{-1}$ kinetin proved to be most suitable medium, for callus induction, thus confirming Shojaei *et al.*, (2010) results. In case of hypocotyls and cotyledons an MS medium containing $2.5mgl^{-1}$ BAP and $3mgl^{-1}$ 2,4-D and one containing 2.0 mgl^{-1} BAP and 2.5 mgl^{-1} 2,4-D was proved to be most efficient, which was used for the first time, for efficient regeneration of spinach from cotyledons and hypocotyls explants. Explant type proved to have significant impacts on regeneration thus confirming Geekiyanage *et al.*, (2006) and Sasaki (1989) results. As kanamycin is inhibitory to spinach growth (Al-Khyari, 1995), calluses were transferred to MS medium lacking kanamycin immediately after selection.

PCR analysis of genomic DNA extracted from transformed and non-transformed (control) calli confirmed the successful integration of *NIC* gene. Using the primers for the *NIC* gene, amplified fragment (1.2kb) of the *NIC* was obtained which showed that the gene of interest is integrated in the spinach genome. *NIC* gene encodes a synthetic chitinase (an antifungal protein), whose expression in spinach is believed to confer resistance to phytopathgenic fungi, because chitinases play a role in defending plants against fungal infections (Carr & Klessig, 1989; Boller, 1988).

In the present study we have just developed transgenic spinach calli containing *NIC* gene for the first time. Since cold temperature (14^oC) is required for shoot regeneration in spinach, due to repeated electric failures, proper shoot regeneration could not be achieved in the present studies. Hence, it is recommended that shoot regeneration may be achieved in further studies under controlled conditions. Further, molecular analysis is suggested to get the final transgenic spinach plants.

In this study we used a synthetic chitinase gene named as *NIC* (Nakamura Ikuo Chitinase) which encoded an identical amino acid sequence to that of chitinase 1 in *R. oligosporus* (Yanai *et al*., 1992) but its nucleotide sequence was designed to adjust to codon usages of plants. The Chitinase 1 (*Chi* 1) protein has been reported to exhibit enhanced resistance against *B. cinerea* in transgenic tobacco (Terakawa *et al.*, 1997). Therefore, it is expected that *NIC* gene exhibit high chitinolytic activity against the cell walls of *R. oligosporus* as well as those of phytopathogens. Recently, transgenic petunia with the *NIC* transgene showed enhanced resistance against *Botrytis cinerea* (Khan *et al.*, 2012). The transgenic spinach in the present study need to be further carried out to get the final transgenic plants and confirmed for the *NIC* transgene by molecular analyses and also to be assayed for their potential disease resistance.

Conclusions and recommendations: In the present study, we have tried to develop an efficient protocol for transformation of spinach with as Synthetic Chitinase gene (*NIC*). Leaves, hypocotyls and cotyledons were used for transformation. Leaf explants proved to be the most efficient for transformation as well as successful callus induction. Growth regulators such as Benzyl Amino Purine (BAP) and 2,4-D were used in combination for the first time for regeneration of hypocotyls and cotyledon explants and proved to be an efficient combination for callus induction from the above mentioned explants. An infection time of 10-15 minutes with *Agrobacterium* inoculums having optical density of 0.9, followed by co-cultivation of 72 hours is recommended for efficient transformation of spinach. In current research, we have just developed transformed calli, shoot regeneration, rooting and further molecular analysis is recommended to develop final transgenic plants.

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