Ann Microbiol (2013) 63:669–675
DOI 10.1007/s13213-012-0517-5

ORIGINAL ARTICLE

Overexpression of recombinant Nep1 in *Escherichia coli* and its use as a biological agent for control of *Sinapis arvensis*

Hamid Moghimi · Javad Hamedi · Zargham Sepehrizadeh · Hamideh Ofoghi

Received: 11 April 2012 / Accepted: 19 July 2012 / Published online: 5 August 2012

Abstract Weeds are a significant part of the pests limiting crop production. Currently, chemical herbicides are widely used for weed control. Environment pollution and the rise of resistant strains highlight the need for new herbicides. Nep1 is a natural bio-herbicide protein which is an effective necrosis stimulant in dicotyledonous weeds. In this study, the cDNA encoding *nep1* was isolated form *Fusarium oxysporum*, cloned and overexpressed in *Escherichia coli.* The Nep1 inclusion body was purified and refolded. For biological assay, the recombinant Nep1 was applied on *Sinapis arvensis,* as a chemical herbicide-resistant weed, and on *Nicotiana tabacum*, as a model plant. Our results show a significant necrosis on the leaves of *S. arvensis* and *N. tabacum* after spraying 50 μg/ml of the recombinant protein.

H. Moghimi · J. Hamedi (✉)
Microbial Biotechnology Laboratory,
Department of Microbiology,
School of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran,
Tehran, Iran
e-mail: jhamedi@ut.ac.ir

H. Moghimi
e-mail: moghimi@khayam.ut.ac.ir

H. Moghimi · J. Hamedi
UTMC, University of Tehran Microorganisms Collection,
Tehran, Iran

Z. Sepehrizadeh
Department of Pharmaceutical Biotechnology,
Faculty of Pharmacy, Biotechnology Research Center,
Tehran University of Medical Sciences,
Tehran, Iran

Z. Sepehrizadeh
e-mail: zsepehri@tums.ac.ir

H. Ofoghi
Biotechnology Department,
Iranian Research Organization for Science and Technology (IROST),
Tehran, Iran
e-mail: hamideh2@yahoo.com

Keywords Bio-herbicide · Expression · Necrosis and ethylene-inducing protein · Recombinant protein · *Sinapis arvensis*

Introduction

Weeds are the main pest affecting crop production and more than 60 % of utilized pesticides are herbicides. Each year, growth of the weeds causes yield loss of total crop production by ~9.7 % in the world (Li et al. 2003). Application of chemical herbicides is the main approach to weed control. However, it has negative consequences on human and animal health and the environment, such as contamination of soil, groundwater and food, the killing of non-target organisms and the development of herbicide-resistant weeds (Chutia et al. 2007). Bio-herbicides are considered as an alternative powerful tool for weed management. A high degree of specificity towards the target weed, safety for the environment and reduction in the development of weed resistance are the main advantages of bio-herbicides (Saxena and Pandey 2001). Bialaphos and phosphinothricin as natural phytotoxins, as well as fungal spores of *Colletotrichum gloeosporioides, Alternaria cassia* and *Alternaria* sp., are examples of commercialized natural herbicides (Hoagland et al. 2007).

Bio-herbicides are screened and selected between plant pathogens, including bacteria, fungi and insects that widely infect weeds (Hoagland et al. 2007). However, the application of bio-herbicides on raw crops has different consequences. Environmental conditions have important roles in the effectiveness of bio-herbicides. For instance, fungal spores need 100 % humidity for germination, which can rarely be found in the field (Amsellem et al. 2002).

Phytotoxins of pathogenic fungi such as necrosis and ethylene-inducing protein1 (Nep1) have also been considered as natural alternatives for some conventional synthetic

herbicides. Nep1 was first isolated from *Fusarium oxysporum* and contains 253 amino acids, including a 31 amino acid signal peptide (Bailey 1995). Many other Nep1-like proteins (NLPs) have been discovered in a variety of microorganisms. They range from 24 to 26 kDa and contain a conserved motif of seven amino acids: GHRHDWE (Gijzen and Nürnberger 2006). NLPs are produced by a variety of taxonomically unrelated microorganisms, including several fungi and oomycetes, as well as Gram-negative and Gram-positive bacteria (Gijzen and Nürnberger 2006). These proteins act as a contact bio-herbicide and penetrate into the leaf through the stomata, rapidly killing the surrounding cells in all dicotyledonous plants. However, they have no effect on monocotyledonous plants (Bailey et al. 2000a; Ottmann et al. 2009). NLPs have been intended for application as bio-herbicides in soil cultivated with monocotyledonous crops, such as wheat, corn, barley and rice (Bailey et al. 2000b). It has also been demonstrated that a combination of Nep1 with the chemical herbicides glyphosate and 2, 4-dichlorophenoxy acetic acid accelerates necrosis development in *Centaurea maculosa* (Bailey et al. 2000a). Nep1 in combination with the bio-herbicidal plant pathogen *Pleospora papaveracea* has been applied to enhance control of *Papaver somniferum*. In addition, transformation of the bio-herbicide fungus *Colletotrichum coccoides* with *nep1* increased the fungus virulence more than nine-fold and caused rapid death when inoculated onto seedlings of *Abutilon theophrasti* (Amsellem et al. 2002).

An *E. coli* expression system is a commonly used model for production of recombinant proteins. Low costs, fast cell proliferation, simple culture conditions and high target gene expression are some of the advantages for protein expression in *E. coli* (Boghigian and Pfeifer 2008).

In this study, we produced a recombinant bio-herbicide Nep1 in *E. coli* and evaluated the activity of the purified protein on wild mustard and tobacco. Bio-herbicide activity of Nep1 on various weeds has been reported previously (Bailey et al. 2000b), but, to our knowledge, this is the first report on high expression recombinant *nep1* and its application on wild mustard, a weed resistant to common chemical herbicides.

Materials and methods

Culture conditions and extraction of total RNA

Fusarium oxysporum f. sp. *lycopersici* UTMC01733 (from the University of Tehran Microorganisms Collection) was cultured in Czapek-Dox broth plus 1 % casamino acids at 30 °C and 200 rpm. After 4 days, 200 mg of centrifuged mycelia was frozen in liquid nitrogen and immediately ground using a mortar and pestle under highly denaturing conditions (guanidine isothiocyanate 5.4 M, pH 6.5). The cell lysate was centrifuged at 10,000 *g*. Total RNA was extracted from the supernatant by RNeasy Plant Minikit (Qiagen, Germany) following the manufacturer's instruction. The quality and quantity of extracted RNA was monitored by denaturing agarose gel electrophoresis and spectrophotometric methods.

cDNA synthesis and RT-PCR

The extracted RNA was used as a template to synthesize single-strand cDNA using the cDNA synthesis system (Roche, Germany) according to the manufacturer's instructions. The synthesized cDNA was used as a template for amplification of *nep1* without signal peptide. The location of the Nep1 signal peptide cleavage site was predicted by SignalP 4.0 Server (www.cbs.dtu.dk/services/SignalP/). Gene-specific sense primer (5′-CAT ATG GCC GTA GTT AAC CAT-′3) with an *Nde*I recognition site (underlined) and an anti-sense primer (5′-GGA TCC TCA GGA CCA GGC CTT-′3) containing a *Bam*HI recognition site (underlined) were used for PCR. All PCR amplifications were done with Pwo Master Polymerase (Roche, Germany). The PCR program was as follows: double-stranded DNA denaturation at 94 °C for 30 s, primer extension at 72 °C for 90 s, and annealing for 30 s at 60 °C for 30 cycles. The amplified PCR product was sequenced (Macrogen, South Korea) and analyzed by Blast software (NCBI).

Cloning of Nep1

The PCR product was blunt ligated to pUC19 and transferred into *E. coli* Nova-blue using the Gene pulser Xcell (2.5 kV, 25 μF, 200Ω). Positive white colony selection was performed in LB medium (1 % Tryptone, 0.5 % yeast extract, 0.5 % NaCl) contain X-gal, Isopropyl-β-D-thiogalactoside (IPTG) and ampicillin. *pUC19-nep1* was extracted and cut by *Nde*I and *Bam*HI. Gene fragment was gel extracted and ligated into the identically cleaved pET16b. *E. coli* BL21 (DE3) strain was transferred using the Gene pulser Xcell (2.5 kV, 25 μF, 200Ω). The transformed cells were cultured on LB agar medium containing ampicillin (100 μg/ml) and the colony selection was performed by PCR. Finally, recombinant plasmid *pET16b-nep1* was purified and sequenced.

Expression, purification and refolding of recombinant Nep1

The recombinant pET16b-*nep1* transformants *E. coli* BL21 (DE3) were cultured in LB medium containing ampicillin (100 μg/ml), up to OD 0.6 at 600 nm. Then, 0.1–1.5 mM IPTG was added to induce expression of the cloned gene. Samples were collected at 0, 1, 2, 3, 4

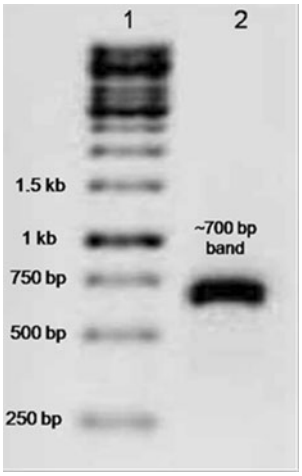

Fig. 1 Agarose gel electrophoresis analysis of DNA fragment amplified by PCR. *Lane 1* Fermentas DNA ladder, *lane 2* the RT-PCR amplified fragment of *nep1* without fungal signal peptide

and 5 h intervals of induction. The biomass was harvested by centrifugation and cells were lysed by a lysis buffer containing 100 μg/ml lysozyme. The insoluble fraction was collected by centrifugation at 10,000 *g* for 10 min at 4 °C and was separated from the soluble fraction. Recombinant protein was purified using nickel-nitrilotriacetic acid agarose (Ni-NTA) kit (Qiagen) according to the manufacturer's instructions. The insoluble fraction containing recombinant Nep1 as an inclusion body was refolded using the Protein Refolding Kit (Novagen, Germany) according to the manufacturer's instructions. For Nep1 refolding, whole-cell pellets were solubilized in a solution containing CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 11) in combination with N-lauroylsarcosine (as a separate agent) and Dithiothreitol (as a reducing agent) to achieve solubility of the inclusion bodies. Finally, the samples were refolded by dialysis against a buffer containing 20 mM Tris-HCl, pH 8.5, in order to remove denaturing and reducing agents. Refolded protein concentrations were determined using the Bradford protein assay with human serum albumin as a standard. The expression and purification of recombinant protein was monitored by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The non-recombinant pET16b transformants were used as the blank sample in the above-mentioned processes.

Biological assay of Nep1

Effects of recombinant Nep1 on plants were studied on wild mustard (*Sinapis arvensis),* as a common weed, common wheat (*Triticum aestivum)* as a monocotyledon model and *Nicotiana tabacum,* as a common dicotyledon. The sterilized seeds of *S. arvensis* and *T. aestivum* were cultivated in Murashige and Skoog (MS) medium with B5 vitamin, in a growth chamber with controlled temperature (27:20 °C day: night) and light cycle (16:8 h light:dark). The seeds of *N. tabacum* were planted in 10-cm pots. All plants were grown outdoors under direct sunlight. Six weeks after planting, mature whole plants were used for biological assay. The plants were treated directly with various concentrations of Nep1 (25, 50 and 100 μg/mL) as a foliar spray. Two milliliters of diluted recombinant Nep1 was sprayed for each plant. As a control, another group of plants were treated by buffer (10 mM Tris-HCl, pH 8.5). All sprays were applied between 10:00 and 14:00 hours when stomata were fully open. Each treatment was done in triplicate in two independent runs.

Results and discussion

Isolation of *nep1* and construction of expression vector

The Nep1 coding sequence from *F. oxysporum* consists of two exons interrupted by a single 58-nucleotide intron (Ottmann et al. 2009). One set of specific primers, with the sequences needed for the restriction endonucleases *Nde*I and *Bam*HI, was designed to amplify *nep1* c-DNA. RT-PCR products were

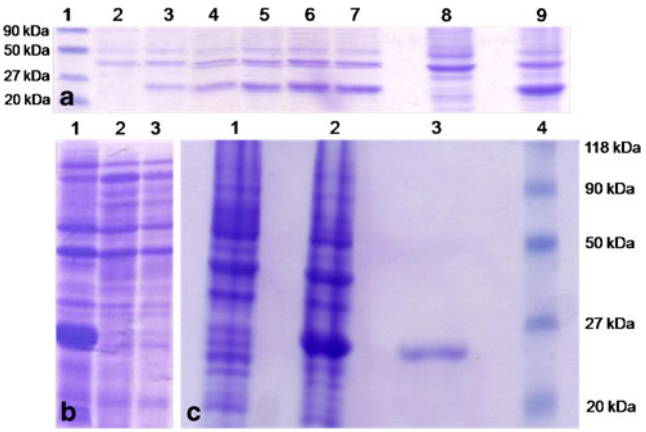

Fig. 2 Coomassie-Blue-stained SDS-poly acrylamide gel analysis of recombinant Nep1 overexpressed in *E. coli*. **a** Analysis of total protein from a culture of *E. coli* BL (DE3) containing *pET16b-nep1* at different times after induction with 1 mM IPTG. *Lane 1* protein size marker, *lane 2* total protein analysis befor induction, *lanes 3–7* total protein analysis after induction of 1 to 5 h, respectively. *Lane 8* whole cell protein from *E. coli* BL (DE3) containing pET16b as a negative control, *lane 9* whole cell protein from *E. coli* BL (DE3) containing *pET16b-nep1* as a positive control. **b** SDS-PAGE of insluble (*lane 1*), soluble (*lane 2*), fractions of recombinant *E. coli pET16b-nep1* and (*lane 3*) total protein from *E. coli* BL (DE3) containing pET16b as a negative control protein. **c** SDS-PAGE analysis of Ni-NTA of recombinant Nep1. *Lane 1* total protein from *E. coli* BL (DE3) containing pET16b as a negative control, *lane 2* total protein from *E. coli* BL (DE3) containing *pET16b-nep1*, *lane 3* recombinant purified Nep1, and *lane 4* protein size marker

obtained with total RNA extracted as a template. A purified 669-bp DNA fragment was sequenced (Fig. 1). The predicted Nep1 is composed of 222 amino acids with a calculated molecular mass of 24 kDa. The isolated sequence from *F. oxysporum* f. sp. *lycopersici* showed 99 % homology with isolated *nep1* from *F. oxysporum* f. sp. *erythroxyli* (Genbank: AF036580) (Bailey et al. 2000a).

Expression, purification and refolding of Nep1

The expression of Nep1 was induced under different induction times, temperatures and Isopropyl-β-D-thio-galactoside (IPTG) concentrations. The optimal induction time was about 4 h (Fig. 2a); there was no significant improvement by increasing temperature and IPTG concentration (data not shown). However, the optimum induction conditions for Nep1 production were achieved with 1 mM IPTG at 37 °C for 4 h (Fig. 2a). The data presented are representative of three independently experiments. SDS-PAGE analysis of recombinant Nep1 showed a band of about 25 kDa and the results clearly indicated that the target protein was significantly overexpressed in *E. coli.* Densitometric scanning of SDS-PAGE gels determined that 38.7 % of total cellular proteins were recombinant Nep1 (Fig. 2b, c). NLPs of *Botrytis cinerea* were expressed and fluorescently labeled in *Pichia pastoris* by Schouten et al. (2008), but the focus of this study was in the mode of action and targeting of NLPs and not in the bio-herbicidal characterization and production yield of recombinant NLPs.

The expressed protein of the foreign gene in *E. coli* can exist in both soluble and insoluble forms (Singh and Panda 2005). The coomassie-stained gels of the soluble and insoluble protein fractions of recombinant *E. coli* demonstrated that, although Nep1 was overexpressed in *E. coli*, a high fraction of the proteins were formed by inclusion bodies which accumulated as insoluble fractions (Fig. 2b). Although protein expression in the form of inclusion bodies is often considered undesirable, it may sometimes be advantageous. The major advantages are high level synthesis of the desired protein, easier purification, and the resistance to cellular proteases (Singh and Panda 2005).

Moreover, designated *pET16b-nep1* contains an N-terminal of 6× His tag upstream of multiple cloning sites for fusing the target protein with 6 histidines. His-tag fusion

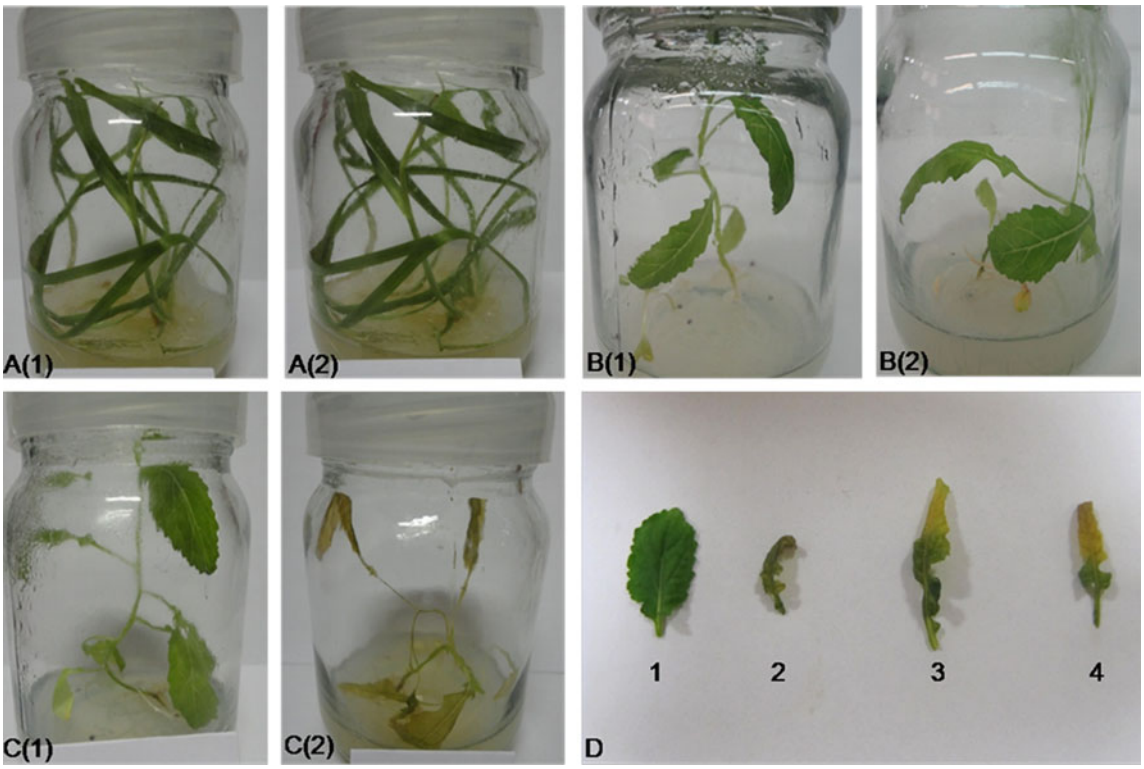

Fig. 3 Biological assay of different concentrations of recombinant Nep1 from *E. coli* in wheat as a monocotyledons model and wild mustard as a common dicotyledonous weed. **A(1)** Cultured wheat before spraying recombinant Nep1, and **A(2)** cultured wheat 5 days after spraying with 50 μg/ml of recombinant Nep1. **B(1)** Cultured mustard before spraying with 20 mM Tris-HCl buffer, and **B(2)** cultured mustard 5 days after spraying with buffer. **C(1)** Cultured mustard before spraying with recombinant Nep1, and **C(2)** cultured mustard 5 days after spraying with 50 μg/ml of recombinant Nep1. **D** Flaccid and necrosed mustard leaves treated with different concentrations of Nep1: *1* untreated, *2–4* treated with 25, 50 and 100 μg/ml of recombinant Nep1, respectively. All the photographs were taken 5 days after treatment. Each treatment was done in triplicate in two separate runs

would facilitate the protein purification with nickel affinity chromatography. The result of purification in different fractions (total lysate of *E. coli* with pET16b-*nep1*, *E. coli* with only pET16b and purified Nep1) was shown (Fig. 2c). Finally, renaturated protein concentration was determined using the Bradford protein assay and stored at −80 °C for biological assay.

Bio-herbicide activity assay of recombinant Nep1

Sinapis arvensis is a common pernicious weed of spring crops, commonly known as wild mustard. This is an aggressive weed, indigenous throughout most of the temperate regions of the world, and representing a serious threat in canola and spring cereals. In spring cereals, dense wild mustard infestations reduce yields by as much as 53 % in wheat, 63 % in oats, and 69 % in barley (Blackshaw and Harker 2002; Lutman 2002).

The most successful technique in controlling wild mustard is to use regular applications of chemical herbicides such as sulfonylurea herbicides and members of the triazine family of herbicides. However, wild mustard is now becoming adapted to many of these herbicides and has become resistant to them (Martin et al. 2001).

Initially, mature wild mustard and wheat were sprayed with different concentrations of Nep1. The symptoms started to appear within 48 h, when the tissue dried out and its color turned to yellowish brown. Maximum necrosis extension was observed after 5 days in wild mustard (Fig. 3c, d). Widespread necrosis was apparent after treatment of leaves with different concentrations of Nep1, and necrosis spread throughout the entire leaves. In the negative control plants, no symptoms were observed during the time considered for the experiment (Fig. 3a).

Moreover, 50 μg/ml of purified Nep1 was sprayed on 6-week-old tobacco plants which were quite sensitive to Nep1 as they started to show necrosis within 24 h. Five days after treatment, mature vegetative tissue was severely damaged and the margin of the leaves turned dark brown and whole leaves necrosed and dessicated (Fig. 4c–e). A negative control monocotyledonous plant, common wheat, was treated with Nep1 (Fig. 3a), while another control was a dicotyledon sprayed with buffer (as a positive control) (Fig. 4). Each treatment was done in triplicate in two separate runs.

According to these results, 48 h after plant treatment with recombinant Nep1, the leaves of *S. arvensis* became flaccid and dull green, due to rapid water loss (Figs. 3, 4). These results are consistent with previous reports showing that

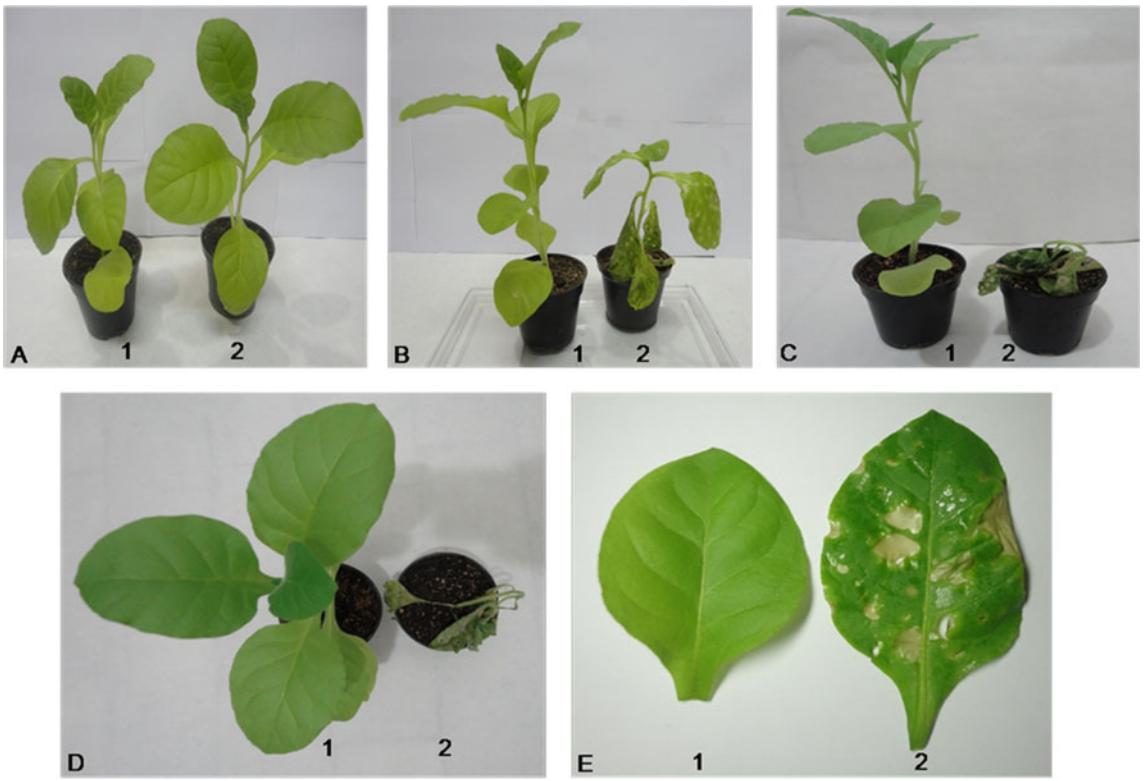

Fig. 4 Necrosis induced in tobacco plants by spraying with 50 μg/ml the recombinant Nep1. In all panels, *1* is the negative control sprayed with Tris-HCl buffer and *2* is the plant treated with the recombinant protein. The photographs **A**, **B**, **C** and **D** were taken 0, 2, 4 and 5 days after treatment, respectively. **E** Leaves of a 6-week-old tobacoo plants sprayed with Tris-HCl as control (*1*) and Nep1 50 μg/ml (*2*), at 2 days after treatment. Each treatment was done in triplicate in two separate runs

foliar application of native Nepl causes membrane destruction (Dallal Bashi et al. 2010; Motteram et al. 2009). However, Nep1-treated wheat leaves, as a monocotyledonous plant, did not develop any necrosis (Fig. 3a). Necrosis signs were seen in tobacco and wild mustard on the first and second days, respectively. However, 100 % of the plants were necrosed 5 days after treatment. The obtained results proved that activity of the recombinant Nep1 was exactly identical to native purified protein (Bae et al. 2008).

It has been reported that the amount of Nep1 required to induce necrosis in plant leaves is about 5–10 μg/ml (Bailey et al. 2000b). However, our results show that, although spraying of Nep1 at concentrations lower than of 25 μg/ml induces necrosis, in order to obtain a systemic necrotization in whole plant, higher concentrations of recombinant Nep1 should be used (Figs. 3, 4). This is consistent with our knowledge that spraying with 25, 50 and 100 μg/ml of Nep1 causes severe damage in all leaves and stems (data not shown). However, the best results were obtained with 50 μg/ml (Figs. 3, 4).

Necrosis ratings for all tested plants in this study were 100 % and no necrosis signs were detected in the control plants. In the similar study, Bailey et al. (2000b) showed that a culture filtrate of native Nep1 from *F. oxysporum* caused necrosis on opium poppy leaves. The necrosis ratings for plants treated with native Nep1 were between 60 and 95 % 7 days after treatment (Bailey et al. 2000b). These results demonstrated that the recombinant protein produced was completely active in greenhouse experiments.

Bio-pesticides are very important transitional tools for organic farming (Keates et al. 2003). Therefore, recombinant Nep1 can be used in a closed organic system situation, because organic farmers currently have limited weed control choices. To our knowledge, this is the first report of biological control of *S. ardencies* in greenhouse conditions. On the other hand, it is difficult to extrapolate the present results to the field, although by estimating the area of pots and the optimum concentration used of Nep1, it can be concluded that approximately 200 g/ha of recombinant Nep1 would be needed to control wild mustard. Imazamethabenz-methyl has been successfully applied at 350–500 g/ha to control *S. arvensis* (Frisen and Wall 1991). In addition, Nepl remained active when co-applied with the chemical herbicides glyphosate and 2,4-dichlorophenoxy acetic acid (Bailey et al. 2000a). The advantages of this synergism are the reduction of chemical herbicide usage and an increase in activity. Today, the cost of mass producing recombinant Nep1 is high, but optimization of up-stream and down-stream parameters markedly reduces the cost of biotechnological products, such as recombinant proteins (Demain and Vaishnav 2009).

In conclusion, we successfully cloned and overexpressed *nep1* heterologously in *E. coli*, and its activity was positively evaluated in wild mustard, a ubiquitous weed resistant to chemical herbicides. This investigation provides a prokaryotic expression system with high efficiency, giving a product of simple purification and of great potential for scale-up. We hope that this recombinant protein will be useful for bio-herbicide development.

Acknowledgments The authors would like to thank Dr. Ali Mohammad Latifi for his valuable support.

References

Amsellem Z, Cohen BA, Gressel J (2002) Engineering hypervirulence in a mycoherbicidal fungus for efficient weed control. Nat Biotechnol 20:1035–1039

Bae H, Kim SH, Kim MS, Sicher RC, Lary D, Strem MD, Natarajan S, Bailey BA (2008) The drought response of *Theobroma cacao* (cacao) and the regulation of genes involved in polyamine biosynthesisby drought and other stresses. Plant Physiol Biochem 46:174–188

Bailey BA (1995) Purification of a protein from culture filtrates of *Fusarium oxysporum* that induces ethylene and necrosis in leaves of *Erythroxylum coca*. Phytopathology 85:1250–1255

Bailey BA, Collins R, Anderson JD (2000a) Factors influencing the herbicidal activity of Nep1, a fungal protein that induces the hypersensitive response in *Centaurea maculosa*. Weed Sci 48:776–785

Bailey BA, Apel-Birkhold PC, Akingbe OO, Ryan JL, O'Neill NR, Anderson JD (2000b) Nep1 proteinfrom *Fusarium oxysporum* enhances biological control of opium poppy by *Pleospora papaveracea*. Phytopathology 90:812–818

Blackshaw RE, Harker KN (2002) Selective weed control with glyphosate in glyphosate-resistant spring wheat (*Triticum aestivum*) 1. Weed Technol 16:885–892

Boghigian BA, Pfeifer BA (2008) Current status, strategies, and potential for the metabolic engineering of heterologous polyketides in *Escherichia coli*. Biotechnol Lett 30:1323–1330

Chutia M, Mahanta J, Bhattacharyya N, Bhuyan M, Boruah P, Sarma T (2007) Microbial herbicides for weed management: prospects, progress and constraints. Plant Pathol J 6:210–218

Dallal Bashi Z, Hegedus DD, Buchwaldt L, Rimmer SR, Borhan MH (2010) Expression and regulation of *Sclerotinia sclerotiorum* necrosis and ethylene-inducing peptides (NEPs). Mol Plant Pathol 11:43–53

Demain AL, Vaishnav P (2009) Production of recombinant proteins by microbes and higher organisms. Biotechnol Adv 27:297–306

Frisen GH, Wall DA (1991) Control of *Sinapis arvensis* in safflower with postemergence. Crop Prot 10:74–77

Gijzen M, Nürnberger T (2006) Nep1-like proteins from plant pathogens: recruitment and diver sification of the NPP1 domain across taxa. Phytochemistry 67:1800–1807

Hoagland RE, Boyette CD, Weaver MA, Abbas HK (2007) Bioherbicides: research and risks. Toxin Rev 26:313–342

Keates SE, Kostman TA, Anderson JA, Bailey BA (2003) Altered gene expression in three plant species in response to treatment with Nep1, a fungal protein that causes necrosis. Plant Physiol 132:1610–1622

Li Y, Sun Z, Zhuang X, Xu L, Chen S, Li M (2003) Research progress on microbial herbicides. Crop Prot 22:247–252

Lutman P (2002) Estimation of seed production by *Stellaria media, Sinapis arvensis* and *Tripleurospermum inodorum* in arable crops. Weed Res 42:359–369

Martin SG, Van Acker RC, Friesen LF (2001) Critical period of weed control in spring canola. Weed Sci 49:326–333

Motteram J, Küfner I, Deller S, Brunner F, Hammond-Kosack KE, Nürnberger T, Rudd JJ (2009) Molecular characterization and functional analysis of MgNLP, the sole NPP1 domain-containing protein, from the fungal wheat leaf pathogen *Mycosphaerella graminicola*. Mol Plant Microbe Interact 22:790–799

Ottmann C, Luberacki B, Küfner I, Koch W, Brunner F, Weyand M, Mattinen L, Pirhonen M, Anderluh G, Seitz HU (2009) A common toxin fold mediates microbial attack and plant defense. Proc Natl Acad Sci USA 106:10359–10364

Saxena S, Pandey AK (2001) Microbial metabolites as eco-friendly agrochemicals for the next millennium. Appl Microbiol Biotechnol 55:395–403

Schouten A, Baarlen PV, van Kan JAL (2008) Phytotoxic Nep1-like proteins from the necrotrophic fungus *Botrytis cinerea* associate with membranes and the nucleus of plant cells. New Phytol 177:493–505

Singh SM, Panda AK (2005) Solubilization and refolding of bacterial inclusion body proteins. J Biosci Bioeng 99:303–310