INTRODUCTION OF FUNGAL NECROSIS INDUCING PHYTOTOXIN FOR BIOCONTROL OF *SINAPIS ARVENSIS* AS A COMMON WEED IN IRAN

F. Shakeri[1], L. A. Mohammad[1], M. Mirzaii[1], H. Babavalian[2] and H. Tebyanian[1]

Applied Biotechnology Research Center, [2]Applied Virology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran
*Corresponding authors E-mail: tebyan.hamid@yahoo.com

ABSTRACT

Weeds are always a serious problem in agriculture. Depending on the type of product and weed, wide ranges of fighting methods have been utilized and consequently, it is associated with different harmful economic and environmental effects in different countries. *Sinapis arvensis* is a common weed with the ecumenical expansion causing performance reduction of many crops, especially wheat. In comparison to the other methods, the biological control method is more efficient. In this study, 4 strains of pea pesto-stricken farms, were isolated from Iran - Lorestan with the aim of biological control of the *Sinapis arvensis* weeds. The effects of 50 µl /cm2 produced supernatant of 7 days cultured fungal biomass that was purified by filtration were assayed, on the goal plant in laboratory and research greenhouse, three-fold and according to numbering method, the most necrotic was related to *IRLM.LC$_{34}$* strain, because destructive effects were appeared as necrosis on leaf, stem and finally lead to the death. Therefore this strain was selected for further study. Since the strain was native, the supernatant was inspissate using ammonium sulfate, as a result, causes severe destructive effects on the plant. The protein purification using FPLC showed the presence of the main part containing a protein with about 24 kDa. The direct effect of 50 µl purified protein mixed with 0.5% tween 20 on the leaves of weed, resulting in necrosis on target plant. Its morphological properties survey based on 18S rDNA analysis showed that this strain has the most similarity to *Fusarium exosporium.* These results indicated that selective destructive effect on dicotyledon weeds and non-harmful effect on the type monocotyledon plants like wheat. In addition, losing of the biological activity of phytotoxin could have any serious environmental hazard.

Keywords: Phytotoxin; Biological Control; *Sinapis arvensis*; FPLC; *Fusariumoxysporum.*

INTRODUCTION

Weeds are as permanent companions of crop plants and the main reasons for the difficulties of agricultural development and reduction of crop products(Mohassel *et al.* 2011, Shakeri *et al.* 2014). There are approximately 30 thousand types of weed in the world that the losses to crops by 1800 types is 7.9 percent of total annual products (Li *et al.* 2003, Chutia *et al.* 2007). Cereal is considered as a strategic and critical product and in comparison to the other Cereal, Wheat is considered as the most important supplier of human food needs in the world, especially in developing countries (Haggui *et al.* 2006). *Sinapis arvensis* is one of the important weeds in cereal crops. This plant is introduced as a weed of 30 crops in 52 countries, and due to rapid growth, extensive root system and an increase in leaf surface for the absorption of nutrients, water and light are considered as a tough competitor (Macák *et al.* 2008). One of the common methods of dealing with *Sinapis arvensis* is the use of chemical herbicides. But due to many problems created such as the appearance of resistant weeds to chemical herbicides (Guzzella *et al.* 2006, Shakeri *et al.* 2014) and environment pollution, scientists have led the studies the biological herbicide, with two main characteristics of relatively short half-life and non-toxic (Hassanshahian *et al.* 2012, Hassanshahian *et al.* 2013, Tebyanian *et al.* 2013, Shakeri *et al.* 2014, Karim Salmani *et al.* 2016, Makzum *et al.* 2016, Rasooli *et al.* 2016, Zarparvar *et al.* 2016) to non-target organisms. Biological herbicides include Plant pathogenic microorganisms or microbial compounds from Pathogenic and non-pathogenic microorganisms that are called phytotoxin (Chutia *et al.* 2007). Microbial phytotoxins are derivatives of bacterial, fungal and actinomycetes (Li *et al.* 2003, Amusa 2006). In recent years, a large number of plant pathogens (bacteria and fungi) and allelochemical compounds are isolated, identified and the herbicide ability is studied (Hoagland 2001).

Nowadays the use of fungal phytotoxins to control weeds, which cause problems, is more common (Punzo 2009). This is probably a result of the stronger effect or better-known fungal pathogens than bacterial ones. Basically, the use of fungi in biological control addition to helping the environment, from economic aspect of consuming of chemical material and toxins, are useful for producers. Are very different in size and chemical structure, Some of them are polypeptides, some are in terpenes groups, And Some other one are

macrocyclic and Bakelite (Evans 2000, Yazdi *et al.* 2016). Among them, peptides are the most important phytotoxic that has a different distribution among the different groups of microorganisms, for illustration hairpin, flagellin, endo-xylanase, syringomycin, NLP and a fungal enzyme such as pectinase and esterase, are the most important of this group. Since phytotoxins protein shows different effects on a certain range of plants, they can be used as Herbicides. Hence the proteins that are effective on the weeds at low concentrations and in the environment condition, Are good candidates for weed control.

In fungal phytotoxins, the type of secreted protein contributes to different symptoms including necrosis (Bailey 1995), chlorosis, languor especially in dicotyledons (Bailey *et al.* 2000). Among the fungal phytotoxin, Necrosis and Ethylene Inducing like Protein is a secretory protein. This protein causes necrosis and the induction of ethylene in many dicotyledonous (Bailey *et al.* 2000, Feng *et al.* 2011). A small assortment of microbial proteins has the ability to activate defense responses and induce necrosis in plant cells through cell signaling pathways (Jennings *et al.* 2000). NLPs are produced by a wide range of microorganisms including a large number of fungal species (Gijzen and Nürnberger 2006) oomycetes, gram positive and negative bacteria (Staats *et al.* 2007). Based on various studies, this protein is used in form of spray or direct injection into the plant and Usually 24h after the entrance of NLP into the vascular system of plant, the signaling cascades initiated within the plant which leads to higher expression of 1-Amino Cyclopropane-1-Carboxylate Synthase and ACC oxidase enzymes, ethylene production, MAP kinase activation, phytoalexin synthesis and intracellular calcium increase, which eventually cause extensive necrosis of plant tissue (Wang *et al.* 2005). Thus, the proteins that are effective on major weeds, at low concentration and environmental conditions, are a good candidate for weeds, considering that most of the weeds are dicotyledonous (Amsellem *et al.* 2002). In order to specific fight against weeds of monocotyledon farm, dicotyledonous weeds can be targeted. Results of this study can be used as a probable alternative to chemical herbicides.

MATERIALS AND METHODS

Fungal isolation: Pest-ridden pea containing the fungal contamination was collected from the chickpea in Lorestan Province. The samples were collected immediately transported to the laboratory and divided into small pieces (2-3 mm) using sterile scissors, the samples were washed with distilled water and transferred to the Petri dishes containing PDA and incubate at 25 ° C for 24 to 48 hours.

Fungal screening for NLP phytotoxin production: The fungal strains purified were incubated with casamino acid at 150 rpm at 28 ^{0}C for 7 days (Shakeri *et al.* 2014). After 7 days, to eliminate mycelium and waste materials, the contents of fungi were passed through a sterile jaconet fabric. The obtained suspension was centrifuged at 10000 rpm for 10 min and the supernatant was filtered through Whitman paper number 1 (Akbar and Javaid 2012).

Sterilization and seeds culture: The seeds were prepared from Medical Plant Research Institute, Tehran. In order to sterilize the seeds, the seeds of *Sinapis arvensis* and *Triticum aestivum* as a negative control (Shakeri *et al.* 2014) were initially placed in 70% alcohol for 30 seconds, then were transferred to a 1% sodium hypochlorite for 3 minutes and followed by washing with sterile distilled water, then the seeds were transferred to 7% agar medium.–After 14 days, the seeds were transferred under sterile conditions into glasses containing Murashige and Skoog medium (Wang *et al.* 2005) in the presence of agar (7%) and pH was adjusted to 7.5 followed by incubation at 20ºC for 20h in the light, at 10ºC for 8h in the dark and 30% humidity. After the forty-fifth day, the young plant is ready to be sprayed by phytotoxin. Seeds were also grown in pots and were kept in greenhouse conditions.

Spraying method: Supernatant of fungal fermentation broth and partially purified mixture protein in the volume of 50 µl/cm2, were sprayed on *Sinapis arvensis* and *Triticum aestivum* with 3 replications, in sterile conditions and with minimal physical stress. MES buffer was used instead of the supernatant and condensed solution, respectively. any necrosis sign was detected every day. Assess the biological activity of the leaves is used because there are no holes (stomata) in the stems and roots. Therefore, all sprays were applied between 10 am and 2 pm.

The effect of the supernatant and condensed solution on weed: Evaluation of the effect of the suspension was performed for several hours after the inoculation of leaves according to the seedling length and the extent of necrosis. The procedure to evaluate the amount of necrosis was as follows; 0, 1, 2, 3, 4, 5 were respectively the diagram of the lack of contamination and 1-25, 26-50, 51-75, 76-99 were the contamination percentage of leaf surface (Ghorbani *et al.* 2000).

Protein precipitation using ammonium sulfate: After the isolation of supernatant, the amount of supernatant was measured, the amount of ammonium sulfate in terms of a gram at 4° C, for 90% saturation and was centrifuged at 21000 rpm for 30 min, then, the supernatant was gently discharged and protein precipitated was kept for the next stage. In order to purify, protein precipitated was dissolved in 20 mM morpholino ethane sulfonic acid monohydrate buffer (pH = 5)and the sample was dialyzed

using dialysis bag with 12000 MW pore in MES buffer to eliminate the waste materials (Bailey *et al.* 2000, Babavalian *et al.* 2016). Since the strain was native, the supernatant was inspissate using ammonium sulfate, as a result, causes severe destructive effects on the plant.

Protein concentration assay: Bradford method was used to detect the protein concentration of the desired sample in the filtered solution (Bailey *et al.* 2000). To calibrate the device, distilled water and MES buffer were used for supernatant and condensed solution, respectively.

Detection of phytotoxic protein by SDS-PAGE: To detect the weight of protein, 12% SDS-PAGE gel and And perpetrated gel was stained by nitrate staining method (Towbin *et al.* 1979, Jennings *et al.* 2001).

Detection of purified protein using chromatography column FPLC: It was performed using the fast protein liquid chromatography technique (AKTA purifier model).For this purpose, 0.1 ml prepared sample was added to1 ml mobile buffer and changes in absorbance (280 nm) was measured. To isolate positively charged proteins, cationic exchange column (sp Toyopearl-m650), 20 mM MES buffer (pH = 5) as buffer A and MES buffer with 1 M KCl as buffer B were used.

To isolate hydrophobic proteins, PhenilToyopearl 650 column, 20 mM MES buffer (pH = 5) as buffer A and MES buffer with 1 mM ammonium sulfate as buffer B were used.

For gel filtration, HW Toyopearl column and 20 mM MES buffer with 1mM KCL (pH = 5) were used. The accuracy of protein function obtained from this method was evaluated on the plant.

Evaluation of the protein purified by FPLC: In order to evaluate protein purified using FPLC, 50 µl purified protein along with 0.5% tween 20 was added on the leaves (Bailey *et al.* 2000, Jennings *et al.* 2001). This experiment was repeated 3 times.

Identification of the strain based on morphology: For primary identification, the strain was cultured in Potato dextrose agar medium and the slide culture method was used to study the microscopic morphology features and the fungal spore chains array.

DNA extraction and 18S rDNA analysis: The genome of *IRLM.LC$_{34}$* fungus was extracted by using glass beads method with some modification (Saghai-Maroof *et al.* 1984). The concentration of DNA extracted was measured using nanodrop and extraction product was detected on gel electrophoresis. 18S rDNAgene was amplified using PCR from *IRLM.LC$_{34}$* genome with forward primer (NU-SSu-0817-5R) 5 TTA GCA TGG AAT AATAAT RRA ATA GGA 3 and reverse primer (NU-SSu-1536R) 5 ATT GCA ATG CYC TAT CCC CA 3.Sequencing of PCR products was carried out with an automated sequencer (Human Genetic Center, Baqyatallah University) (Sanchez-Porro *et al.* 2010, Babavalian *et al.* 2013, Babavalian *et al.* 2014, Tebyanian *et al.* 2014).

Phylogenic tree of *IRLM.LC$_{34}$*: 18S rDNA sequences were compared to other 18S rDNA sequences available in the National Center for Biotechnology Information (NCBI) public database by BLAST searching. After multiple alignments of data available from public databases by CLUSTALW (Thompson et al., 1997), pairwise evolutionary distances and phylogenetic analysis were computed using the Kimura 2 parameters model and UPGMA algorithm in MEGA5 software (Tamura *et al.* 2011, Rasooli *et al.* 2016).

Statistical Analysis: Data obtained from all experiments were analyzed using Spss ver-18 software and Mann-Whitney test was used to compare the groups, furthermore, differences were considered statistically significant at $p<0.05$. Excel 2007 was used to draw graphs.

RESULTS

The effects of phytotoxins on *Sinapis arvensis* for Primary screening: In this study, four strains of the pea plant tissue were extracted and purified. Pathogenicity tests were repeated threefold, and based on the numbering system, the highest scoring rate was 4, related to the virulence of the strain *IRLM.LC$_{34}$*. The strains of *IRLM.LC$_{26}$* and *IRLM.LC$_{12}$* have no significant differences) p<0/05) and *IRLM.LC$_{18}$* strains did not show any signs of disease. Since the aim was to select the most effective strains, therefore, the pathogenic, *IRLM.LC$_{34}$* strain was selected for further study.

The effects of supernatant and the condensed solution of phytotoxins on *Sinapis arvensis*: According to the observed macroscopic effects in the two-leaf stage of *Sinapis arvensis*, the necrosis symptoms had a slow rate from the first day to two days after inoculation, but showed high rate during the other days and approximately 60% of leaf surface was necrosed on the fifth day. The necrosis symptoms began toappearin4-leaf stage after 21hoursand ultimately in day sixth approximately99-75% and in 6-leaf stage 75-51% of leaf surface was necrosed.

The results showed that the growth of 4-leaf stage had the highest sensitivity to the desired protein showed a significant difference in the 2-leaf stage, while no necrosis symptoms were observed in the control plants ($p<0/05$). Average length of the seedling in 4-leaf stage was observed 6.1 cm and 9.8 cm before spray in *Sinapis arvensis* and wheat, and after 6 days after spraying the supernatant, the average length of seedling was observed 11.2 cm which showed 12.5 % increase in the growth toward before spraying and according to the Mann-

Whitney test, there was no significant difference between the test and control (p<0/05). While the *Sinapis arvensis* growth was stopped after 48 h. and seedling length between test and control showed statistically significant difference (Fig. 1). Condensed solution showed a similar effect as the supernatant on *Sinapis arvensis* and wheat, expect that the growth of *Sinapis arvensis* was stopped on the fifth day (Fig. 2). The results also showed that the condensed solution was more powerful compared to the supernatant in the severity of the disease. In experiments conducted in the greenhouse conditions, the effect of phytotoxin from *IRLM.LC34*was also caused necrosis and plant wilting (Fig. 3). This protein is a secretory protein, so, the supernatant was an upper solution which was cultivated from mushrooms cultivation environment and it was condensed by ammonium sulfate and finally was dialyzed. It can be found that this protein had positive effects on cotyledons and it had negative effects on monocots. But, other proteins may have negative effects on monocots and cotyledons.

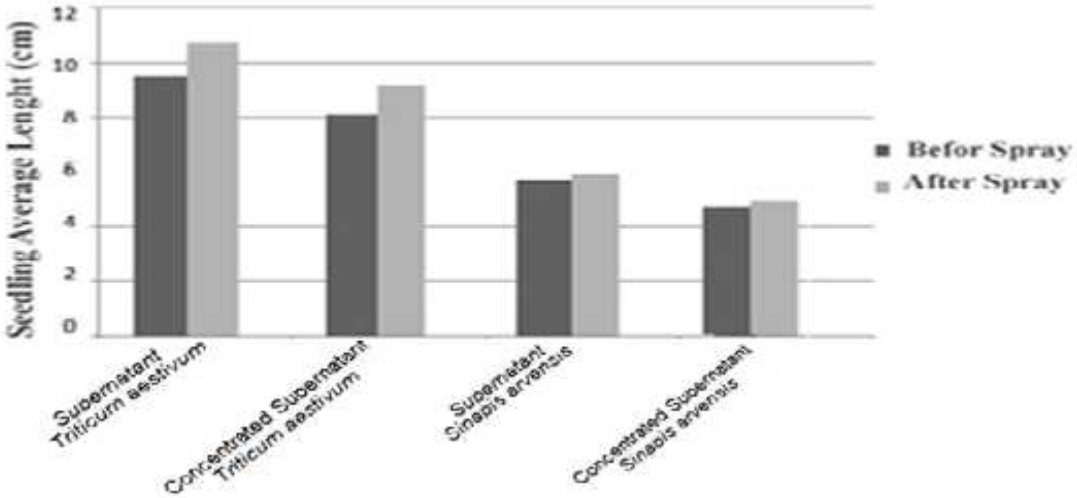

Fig. 1. The average length of seedling after 6 days spraying supernatant and a concentrated solution (after spraying supernatant, the destructive effects on *Sinapis arvensis* started to appear but the wheat showed no reaction to the phytotoxic protein and kept its growth).

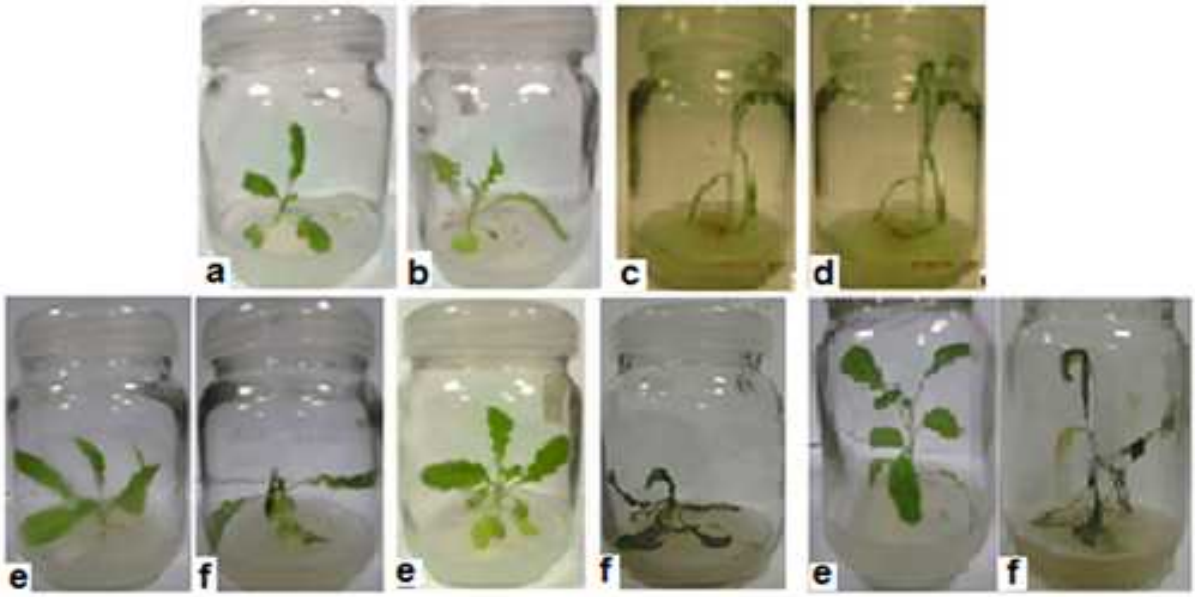

Fig. 2. Control and evaluation of the supernatant of *IRLM.LC34*strain, on*Sinapis arvensis* and wheat, a: Control *Sinapis arvensis*, b: spraying the MES buffer on the *Sinapis arvensis*, c: Control Wheat, d: spraying the *IRLM.LC34* strain on Wheat, e: *Sinapis arvensis* before spraying *IRLM.LC34* strain, f: *Sinapis arvensis* after 6 days of spraying on *IRLM.LC34* strain.

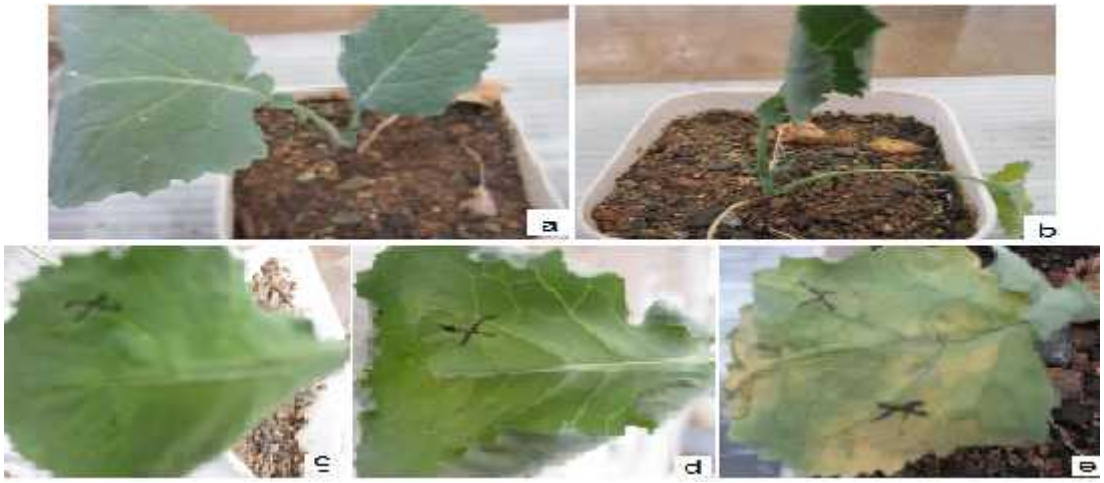

Fig. 3. a. before spraying, b. the twelfth day of spraying, c. a closer look at the leaf before spraying, d. a closer look at the leaf on six days after spraying, e. a closer look at a leaf on twelve days after spraying.

Detection of the concentration and the molecular weight of *IRLM.LC$_{34}$* protein: The protein concentrations of supernatant and condensed solutions were 0.142 mg/ml and 1.017 mg/ml, respectively. The Molecular weight of the proteins of the supernatant and condensed solution is shown on SDS-PAGE gel electrophoresis.

Purification of 24 kDa protein using FPLC: In FPLC method using cationic column, proteins leave the column depending on their ionic strength (Fig. 5), The purification result was obtained using hydrophobic column and based on previous studies, desired protein revealed a peak at about 14 min (Figure 4), The peak of the purified protein of interest by gel filtration column is shown.

Evaluation of the purified protein result: The protein purification using FPLC showed the presence of the main part containing a protein with about 24 kDa. The direct effect of the protein caused necrosis on the target plant after 21 h.

Study of the microscopic and macroscopic morphology of strain: The fungus *IRLM.LC$_{34}$*was morphologically considered as a *Fusarium*, based on the bright color on the back of colony's plate (white with violet flecks) and its apparent form as wooly colonies in white with violet flecks, etc. *IRLM.LC$_{34}$* was also identified to be a member of genus *Fusarium* with Teased mount and slide culture. The identification was based on single and double Chlamydia conidia along the mycelium and also based on that the mycelium and conidia were not stained.

Sequencing and drawing a phylogenic tree of fungal *IRLM.LC$_{34}$*: The results of the18S rDNA sequencing of strain *IRLM. LC$_{34}$*confirmed the morphological obtain results. The results of BLAST analysis of fungal strain *IRLM.LC$_{34}$*revealed 96% similarity to *Fusarium oxysporum* (Figure 5).

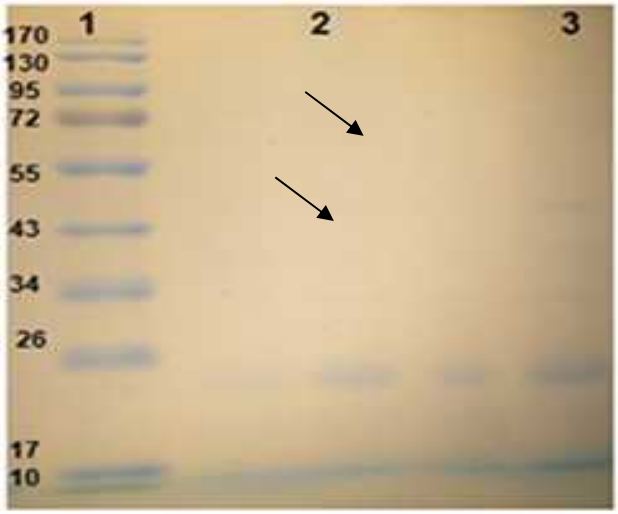

Fig. 4. Detection of 24kDa Protein of *IRLM.LC$_{34}$* strain. Line 1: marker SMO33, Line 2: about 24kDa (between 17 kDa -26 kDa) Band related to the supernatant protein of *IRLM.LC$_{34}$* strain, Line 3: the protein of the concentrated solution using ammonium sulfate, of *IRLM.LC$_{34}$* strain.

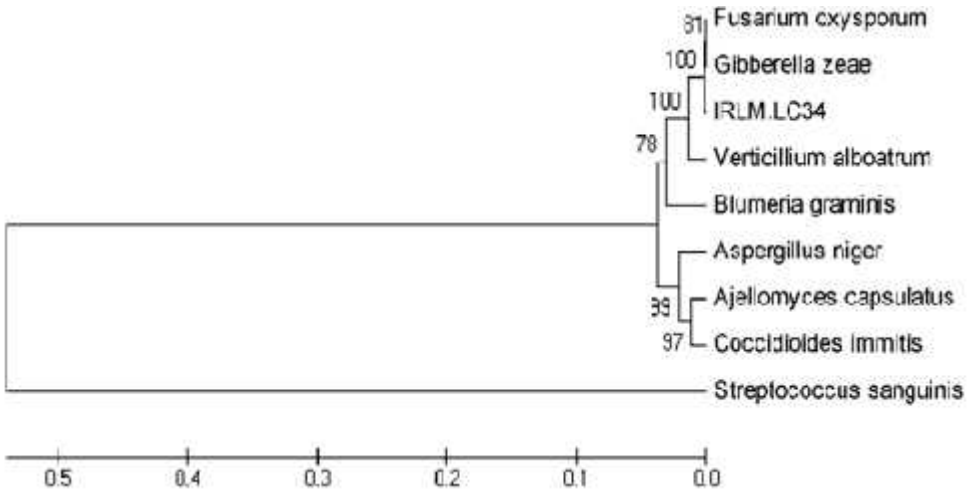

Fig. 5. Phylogenetic tree representing the relationship of the fungal strain isolated from contaminated plant samples (*IRLM.LC34*) with other fungal strains, based on their 18S rDNA . The sequence analysis showed that the isolated strain is probably the same of *Fusarium oxysporum*.

DISCUSSION

Since a variety of effects are observed by protein phytotoxins on a distinct range of plants, they can be used for herbicides property. Hence, the effective proteins on major weeds, at low concentrations and environmental conditions, are good candidates for weeds (Namasivayam 2010).

According to previous studies, most dicotyledon plants are sensitive to 24-26 kDa protein (Schouten *et al.* 2008) and only the type and the level of responses in desired plants are varied, while, studied monocotyledon plants showed no response against this protein (Bailey 1995). The lack of response in monocotyledons can be due to the lack of receptors or the insufficiency of desired protein to reach the function site. In this study, *Sinapis arvensis* was also sensitive to 24 kDa protein, while the wheat showed no sensitivity toward this protein. Different stages of the growth showed a different sensitivity to the protein since the cuticle thickness and the physical strength of leaf compositions probably act as a barrier against pathogens. In this study, the growth of 4-leaf stage showed the highest sensitivity to the protein compared to other stages (Trigiano 2007).

The time of plant necrosis in greenhouse conditions is very long and the necrosis score is less and it may be due to the thickness of cuticle, plant resistance and the lack of sustainability in the supernatant of soil towards the MS medium. Nowadays, different methods are used to condense proteins such as TCA sequestration, acetone sequestration, ultrafiltration, lyophilization (Jiang *et al.* 2004). In this study, in order to purify and evaluate on the plant, ammonium sulfate method was used. In a study by (Garcia *et al.* 2007), the condensed NPP1 protein was used as an inducer of tobacco and the necrosis symptom was observed after 18h, while in this study, necrosis was observed after 21 h. In a study by Bailey (1995) (Bailey 1995), hanging drop method was used to measure necrosis, however, the leaf spray method was used in this study, therefore, the faster absorption with more uniformity and less stress than the other methods was performed.

In more articles, the 24 to 26 kDa protein has reported the cause of necrosis property (Bailey 1995). In this study, the band around 24 kDa was observed on the SDS-PAGE gel; consequently, it was found that this strain is able to eliminate the plant due to the production of desired protein. Hydrophobic properties of the 24 kDa protein are similar to a subgroup of fungal elicitors such as Trichoderma Viride Xylanase and eliciting of Phytophthora strains (Bailey 1995). Therefore, the purification has been cited in all articles to Bailey method (1995) (Bailey 1995). In this study, the gradual changes of buffer concentration were used instead of step-by-step increasing in buffer concentration.

As regards to purpose of experiment, that was finding NLP phytotoxins in 4 isolated of pest-stricken pea plants from province of Lorestan and since obtained phytotoxins were active just on dicotyledon plants, Act extracellular, caused rapid activate of defense cell response and leads cell to necrotic and death (Garcia *et al.* 2007) (since the sequence of this proteins Are not available) These proteins are likely to be one part of the NLP families. According to Bailey study (2000) (Bailey *et al.* 2000), if this protein is applied on leaves along with a strong detergent (to facilitate the penetration) would be a powerful phytotoxin. However, the direct effect of 24-kDa protein purified in this study leads to the necrosis of target plant, its intensity and effects were less than the supernatant and condensed solution. It is concluded that the development of severe necrosis effects, in addition to the protein, involves other factors which remain unknown

at this stage. Results obtained from this study indicate phytotoxic protein as a suitable candidate to control dicotyledon weeds, therefore, can be used as a probable alternative to chemical herbicides.

Acknowledgement: The authors would like to thank all colleagues in the Applied Biotechnology Research Center of Baqiyatallah Medical Sciences University, Analytical Chemistry and Biology departments for their kind contribution to the research.

REFERENCE

Akbar, M. and A. Javaid (2012). Evaluation of herbicidal potential of fungal metabolites against Phalaris minor. African Journal of Microbiology Research. 6(18): 4053-4057.

Amsellem, Z., B. A. Cohen and J. Gressel (2002). Engineering hypervirulence in a mycoherbicidal fungus for efficient weed control. Nature biotechnology. 20(10): 1035-1039.

Amusa, N. A. (2006). Microbially produced phytotoxins and plant disease management. African Journal of Biotechnology. 5(5): 405-414.

Babavalian, H., M. A. Amoozegar, A. A. Pourbabaee, M. M. Moghaddam and F. Shakeri (2013). Isolation and identification of moderately halophilic bacteria producing hydrolytic enzymes from the largest hypersaline playa in Iran. Microbiology. 82(4): 466-474.

Babavalian, H., M. A. Amoozegar, S. Zahraei, R. Rohban, F. Shakeri and M. M. Moghaddam (2014). Comparison of bacterial biodiversity and enzyme production in three hypersaline lakes; Urmia, Howz-Soltan and Aran-Bidgol. Indian journal of microbiology. 54(4): 444-449.

Babavalian, H., A. M. latifi, M. A. Shokrgozar, S. Bonakdar, H. Tebyanian and F. Shakeri (2016). Cloning and expression of recombinant human platelet-derived growth factor-BB in Pichia Pink. Cellular and Molecular Biology. 62(8): 45-51.

Bailey, B. A. (1995). Purification of a protein from culture filtrates of Fusarium oxysporum that induces ethylene and necrosis in leaves of Erythroxylum coca. Phytopathology. 85(10): 1250-1255.

Bailey, B. A., R. Collins and J. D. Anderson (2000). Factors influencing the herbicidal activity of Nep1, a fungal protein that induces the hypersensitive response in Centaurea maculosa. Weed Science. 48(6): 776-785.

Chutia, M., J. J. Mahanta, N. Bhattacheryya, M. Bhuyan, P. Boruah and T. C. Sarma (2007). Microbial herbicides for weed management: prospects, progress and constraints. Plant Pathology Journal. 6(3): 210-218.

Evans, H. C. (2000). Evaluating plant pathogens for biological control of weeds: an alternative view of pest risk assessment. Australasian Plant Pathology. 29(1): 1-14.

Feng, B. Z., P. Q. Li, L. Fu, B. B. Sun and X. G. Zhang (2011). Identification of 18 genes encoding necrosis-inducing proteins from the plant pathogen Phytophthora capsici (Pythiaceae: Oomycetes). Genet Mol Res. 10: 910-922.

Garcia, O., J. A. N. Macedo, R. Tibúrcio, G. Zaparoli, J. Rincones, L. M. C. Bittencourt, G. O. Ceita, F. Micheli, A. Gesteira and A. C. Mariano (2007). Characterization of necrosis and ethylene-inducing proteins (NEP) in the basidiomycete Moniliophthora perniciosa, the causal agent of witches' broom in Theobroma cacao. mycological research. 111(4): 443-455.

Ghorbani, R., W. Seel, A. Litterick and C. Leifert (2000). Evaluation of Alternaria alternata for biological control of Amaranthus retroflexus. Weed Science. 48(4): 474-480.

Gijzen, M. and T. Nürnberger (2006). Nep1-like proteins from plant pathogens: recruitment and diversification of the NPP1 domain across taxa. Phytochemistry. 67(16): 1800-1807.

Guzzella, L., F. Pozzoni and G. Giuliano (2006). Herbicide contamination of surficial groundwater in Northern Italy. Environmental Pollution. 142(2): 344-353.

Haggui, F., P. W. B. Phillips and R. S. Gray (2006). 15 Opposition to Genetically Modified Wheat and Global Food Security. International trade and policies for genetically modified products. 175.

Hassanshahian, M., M. Ahmadinejad, H. Tebyanian and A. Kariminik (2013). Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances). Marine pollution bulletin. 73(1): 300-305.

Hassanshahian, M., H. Tebyanian and S. Cappello (2012). Isolation and characterization of two crude oil-degrading yeast strains, Yarrowia lipolytica PG-20 and PG-32, from the Persian Gulf. Marine pollution bulletin. 64(7): 1386-1391.

Hoagland, R. E. (2001). Microbial Allelochemicals and Pathogens as Bioherbicidal Agents 1. Weed Technology. 15(4): 835-857.

Jennings, J. C., P. C. Apel-Birkhold, B. A. Bailey and J. D. Anderson (2000). Induction of ethylene biosynthesis and necrosis in weed leaves by a Fusarium oxysporum protein. Weed Science. 48(1): 7-14.

Jennings, J. C., P. C. Apel-Birkhold, N. M. Mock, C. J. Baker, J. D. Anderson and B. A. Bailey (2001). Induction of defense responses in tobacco by the protein Nep1 from Fusarium oxysporum. Plant Science. 161(5): 891-899.

Jiang, L., L. He and M. Fountoulakis (2004). Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. Journal of Chromatography A. 1023(2): 317-320.

Karim Salmani, B., M. A. Amoozegar, H. Babavalian, H. Tebyanian and F. Shakeri (2016). Removing Lead from Iranian Industrial Wastewater. International Letters of Natural Sciences 57: 79-88.

Li, Y., Z. Sun, X. Zhuang, L. Xu, S. Chen and M. Li (2003). Research progress on microbial herbicides. Crop Protection. 22(2): 247-252.

Macák, M., S. Zák, M. Birkás, I. Djalovic and P. Slamka (2008). The influence of an ecological and a low input system on yield and yield components of spring barley.

Makzum, S., M. A. Amoozegar, S. M. M. Dastgheib, H. Babavalian, H. Tebyanian and F. Shakeri (2016). Study on Haloalkaliphilic Sulfur-Oxidizing Bacterium for Thiosulfate Removal in Treatment of Sulfidic Spent Caustic. International Letters of Natural Sciences. 57: 49-57.

Mohassel, M. H. R., K. H. Ghalibaf and S. A. Hosseini (2011). Evaluating Some Chemical and Mechanical Weed Management Methods Aiming to Reduce Herbicide Use in Potato (Solanum tuberosum L.). Journal of Plant Protection. 25(3): 29.

Namasivayam, S. K. R. (2010). Herbicidal activity of mycelial and cell free extracts of Fusarium oxysporum F. sp. Ciceris against rice weed Cyperus iria l. Research Journal of Agriculture and Biological Sciences. 6(6): 728-731.

Punzo, B. (2009). Phytotoxins produced by Alternaria sonchi, a potencial mycoherbicide for biocontrol of Sonchus arvensis.

Rasooli, M., M. A. Amoozegar, A. A. Sepahy, H. Babavalian and H. Tebyanian (2016). Isolation, Identification and Extracellular Enzymatic Activity of Culturable Extremely Halophilic Archaea and Bacteria of IncheBoroun Wetland. International Letters of Natural Sciences. 56: 49-57.

Rasooli, M., M. A. Amoozegar, A. A. Sepahy, H. Babavalian and H. Tebyanian (2016). Isolation, Identification and Extracellular Enzymatic Activity of Culturable Extremely Halophilic Archaea and Bacteria of IncheBoroun Wetland. Cellular and Molecular Biology. 62(12): 31-36.

Saghai-Maroof, M. A., K. M. Soliman, R. A. Jorgensen and R. W. Allard (1984). Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proceedings of the National Academy of Sciences. 81(24): 8014-8018.

Sanchez-Porro, C., M. A. Amoozegar, A. B. Fernandez, M. Ramezani and A. Ventosa (2010). Lentibacillus persicus sp. nov., a moderately halophilic species isolated from a saline lake. International journal of systematic and evolutionary microbiology. 60(6): 1407-1412.

Schouten, A., P. Van Baarlen and J. A. L. Van Kan (2008). Phytotoxic Nep1-like proteins from the necrotrophic fungus Botrytis cinerea associate with membranes and the nucleus of plant cells. New Phytologist. 177(2): 493-505.

Shakeri, F., A. M. Latifi, M. Mirzaei, H. Babavalian and F. Hashemlou (2014). Biocontrol of Amaranthus retroflexus and Rumes crispus by NLP phytotoxine, a selective bioherbicide. Journal of Applied Biotechnology Reports. 1(1): pp-44.

Staats, M., P. van Baarlen, A. Schouten, J. A. L. van Kan and F. T. Bakker (2007). Positive selection in phytotoxic protein-encoding genes of Botrytis species. Fungal Genetics and Biology. 44(1): 52-63.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution. 28(10): 2731-2739.

Tebyanian, H., M. Hassanshahian and A. Kariminik (2013). Hexadecane-degradation by Teskumurella and Stenotrophomonas strains isolated from hydrocarbon contaminated soils. Jundishapur Journal of Microbiology. 6(7).

Tebyanian, H., S. H. Mirhosseiny, B. Kheirkhah and M. Hassanshahian (2014). Isolation and Identification of Mycoplasma synoviae From Suspected Ostriches by Polymerase Chain Reaction, in Kerman Province, Iran. Jundishapur journal of microbiology. 7(9).

Towbin, H., T. Staehelin and J. Gordon (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences. 76(9): 4350-4354.

Trigiano, R. N. (2007). Plant pathology concepts and laboratory exercises, CRC Press.

Wang, J.-W., L.-J. Wang, Y.-B. Mao, W.-J. Cai, H.-W. Xue and X.-Y. Chen (2005). Control of root cap

formation by microRNA-targeted auxin response factors in Arabidopsis. The Plant Cell. 17(8): 2204-2216.

Yazdi, H., A. Karami, H. Babavalian, S. A. Mirhosseini and H. Tebyanian (2016). The Effects of some Physicochemical Stresses on Escherichia coli O157: H7 as Clinical Pathogenic Bacteria. International Journal of Agriculture & Biology. 18(6).

Zarparvar, P., M. A. Amoozegar, H. Babavalian, F. M. Reza, H. Tebyanian and F. Shakeri (2016). Isolation and identification of culturable halophilic bacteria with producing hydrolytic enzyme from Incheh Broun hypersaline wetland in Iran. Cellular and molecular biology (Noisy-le-Grand, France). 62(12): 31.