Original Article

Biocontrol of *Amaranthus retroflexus* and *Rumes crispus* by NLP phytotoxine, a selective bioherbicide

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Abstract

Non-beneficial and harmful weeds are plants that are unwanted, outside their home farms are growing and have the potential to exceed. This study was done in order to screening fungai and isolating NLP phytotoxine from them for selective biocontrol of *Amaranthus retroflexus* and *Rumes crispus* as a dicot, common and chemical herbicide resistance weeds. NLPs are effective just on dicot plants. Contaminated soil and dicotyledons plants were Collected from different regions of Iran. after collecting and culturing them, The effect of Supernatant from fungal cultures, was asseyd by spraying of 5 µl /cm3 of it mid 20 µl tween-20 on leaves of *Amaranthus retroflexus,Rumes crispus* and wheat as negative control that were cultured in MS media and pots in 3 replications with completely randomized design in laboratory and research green house of baqiatallah university. The effects were assessed according to numbering method. Finally, the $QAT_5$and G_{7-1}strains was selected from 9 top strains, because was more destructive than others on *Amaranthus retroflexus* and *Rumes crispus* respectively from necrosis to cell death with number 4 according to numbring method and has non-harmful effect on the wheat (*Triticum aestivum*). SDS-page results showed phytotoxine that was produced by QAT_5 strain was a protein and this from G_{7-1} was non-protein. For better result on SDS-page protein was concentrated using by ammonium sulfat method, but about G_{7-1} again this outcomewas repeated. The protein purification of QAT_5strain using FPLC showed the presence of a protein with about 24 kDa like other family members of this protein. Considering this fact that these phytotoxines according to the result had similarity features to what founded befor about NLPs, they are recommended as biocontrol factor of these weeds insteade of chemical herbicides.

Keywords: Biocontrol, *Amaranthus retroflexus*, *Rumes crispus*, selective bioherbicide, *Triticum aestivum*, FPLC

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Introduction

Amaranthus retroflexus is a weed of 60 crops in 70 countries [1], like Wheat, barley, oats, Flax, Rape [2], Canola [3], Corn [4] and so many others. Unfortunately, many Amaranthus species have evolved resistance to herbicides in Canada as well as in many countries world-wide [5, 6]. Heap (2002) reported that six Amaranthus species were among the 25 worst resistant weeds in the world, and ranked *A. retroflexus* number three, based on area infested, number of crops, countries and herbicide modes of action. In Ontario, *A. retroflexus* and *A. powelliiwere* among the first weeds to develop resistance to triazine herbicides, which inhibit photosystem II [8]. *Rumex crispus L.* (curled dock) and *Rumex obtusifolius L.* (broad-leaved dock) are among the most often studied weed species worldwide, the latter is also considered as one of the five most widely distributed non-cultivated plant species in the world [9,10, 11]. Both species are troublesome weeds in both grasslands (mainly pastures) and arable lands, but are also early colonizers of many disturbed areas in lowland and upland. The species are present on almost all soil types but less often on peat and rarely on acid soils. The range of altitude to which these species have become adapted is very great; a maritime ecotype of *R. crispus* grows on beaches another ecotype can also be found at 2500 m above sea level in the Middle East and south-western USA or at 3000 m in Iran and 3500 m in Argentina [10]. Tonev (2000) has listed *Rumex crispus L.* in a group of one hundred economically most important weeds characterized by high biological and ecological plasticity. In crop rotation of lucerne and winter grain cereal, 64% of the weed survives after every ploughing [13]. The importance of successful control of this species has attracted the attention of many researchers [14, 15, 16,17] Beside chemical-resistant weeds problem caused by chemical herbiside [18,19], environmental pollutions by chemicals and inducing allergic reactions in humans and animals caused the scientists to, instead, start their investigations on the use of bio-herbicides which are not toxic to non-target organisms, with a relatively short half-life and appropriate chemical structures to act on molecular sites which are not targets of chemical herbicides [20, 21, 22] to non-target organisms. Phytotoxin produced by contaminating microorganisms is of biological herbicides. Microbial phytotoxins derive from bacterial, fungal and actinomycetes [23, 24]. Currently, fungal phytotoxins are more common in controlling the weeds [25, 26]. This is probably a result of the stronger effect or better-known fungal pathogens than bacterial ones. Among fungal phytotoxins, secreted protein of NLP contributes to different symptoms including necrosis, chlorosis, languor especially in dicotyledons [27, 28, 29], this protein induces a series of plant responses like increase in sensitivity that results more expression of enzymes ACC synthase, ACC oxidase, ethylene production, MAP kinase activation, phytotoxin synthesis and intracellular calcium increase that ultimately causes extensive necrosis of plant tissue [30]. The achievement of the present study can be used as an appropriate candidate in biological weed control and a possible alternative to chemical herbicides.

Materials and methods

Plant samples were collected from various contaminated soil and dicotyledon plants in farms throughout the IRAN like Qazvin, khorram abad, Damavand, Shazand, Abshare bishe and so on by Doctor latifi and collegues. The samples were coded based on the plant and the sampling place, kept in appropriate conditions and transferred to the lab as quickly as possible.

To cultivate seeds of *Amaranthus retroflexus*, *Rumes crispus* and wheat bread (*Triticum aestivum)* as monocotyldon plant [19] that were provided from agronomy biotechnology institute of karaj, the seeds of *Amaranthus retroflexus* and wheat were sterilized in 70% alcohol and 1% sodium hypochlorite and then washed by sterile deionized water and were transferred to Murashige and Skoog medium (MS), then were grown for 16 h light at 20 0C and 8 h dark at 10 0C and 30% humidity. After 45 days the young plants were ready to be sprayed by phytotoxin. In greenhouse planting seeds of *Rumes crispus* and *Amaranthus retroflexus*, they cultured first in trays inside cocopits. After germination, the seedlings were transferred into pots with soil composition (sand, manure, preparated soil leaves and cocopit). Plants were grown under greenhouse conditions (32 days) than that of the seed that were raised in vitro (45 days old) was ready for spray. The isolates were purified in order to study their ability to produce necrosis and ethylen-inducing phytotoxins (NLP) in considered dicotyledons, were incubated in Czapek-Dox broth media with %1 casamino acids in 150 rpm at 28°C for 10 days [31, 19, 32].

The media was centrifuged at 10000 rpm for 10 min in 45 ml tubes for isolation of fungal mass and supernatant was filtered using Whitman paper number 1(improper precipitation of fungi using centrifuge) [32].

After filtering and separation of the supernatant of fungal cultures, the volume of supernatant was measured. Then, it was centrifuged in 21000 rpm for 30 min. afterwards, the supernatant was calmly discarded and the protein precipitate was stored for the next stage.

Supernatant in the volume of 5 µl/cm3, mid 20 µl tween 20 as detergent were sprayed on wheat, *Rumes crispus* and *Amaranthus retroflexus* with 3 replications. In the control treatments (3 replications), czapek medium were used instead of supernatant. Plants were visited daily and any disease symptoms on plants were recorded. The effect of supernatant on the weed was evaluated based on numbering method; 0 = no disease, 1 = 1- 25% infection, 2 = 26 - 50% infection, 3 = 51 - 75% infection, 4 = 76 - 98% infection, 5 = 99% infection (plant death) [33].

Bradford assay was used to determine the total protein concentration [34, 35]. In this stage, the opacity should be read using the spectrophotometer on 595nm wavelength, within 2-20 min. To calibrate the device, distilled water was used for supernatant. The desired protein was detected by SDS-PAGE method. The prepared sample was loaded on 12% SDS-PAGE gel and silver nitrate staining was applied in order to more sharply detect the bands [35, 36].

Study of purified protein from QAT_5strain, positively charged hydrophilic, was performed using FPLC technique (AKTA purifier model). In this method, positively charged proteins was initially isolated using cationic column, and afterward injected into the hydrophobic column, positively charged hydrophilic proteins were purified. Then the gel filtration column was used to separate proteins based on their molecular weight. 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, Phenil Toyopearl 650 column, 20 mM MES buffer (pH = 5) as buffer A and MES buffer with 1 mM ammonium sulphate 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.

Results

With sampling and screening of isolates from the contaminated dicotyledon plants in different regions, 80 pure fungal isolates were obtained. The amount of necrosis by phytotoxin protein was evaluated on the monocotyledon (wheat) and dicotyledonous (*Amaranthus retroflexus* and *Rumes crispus*) samples.

51 of 80 isolates, which were assayed in Czapek medium, inhibited the growth of *Amaranthus retroflexus* and *Rumes crispus*, 0-30% in comparison with the control. 12 and 8 isolates inhibited their growth by 30-50% and 50-70%, respectively, in comparison with the control. Only 9 isolates (L_{2-27}, QAP_1, G_{7-1}, QAT_5,GHB_1, LKD_{8-2},LC_{18},$TKHS_4$,QRP_{1-2}) could inhibit the *Amaranthus retroflexus* and *Rumes crispus* growth by 70% or more. For different reasons, including the destruction efficiency, destruction time, and destruction type and …, $QAT_5$and G_{7-1}strains were selected among the 9 top isolates, and were investigated for future studies. Lack of stomata on stems and roots to measure the biological activity are used on leaves.In the comparison of necrosis, the symptoms of necrosis began to emerge after 24 h and finally lead to complete plant death on the 6 day about *Amaranthus retroflexus* that were cultured in pots (Fig. 1) and in 5 days after spraying about this weed that were cultured in MS media, with number = 4, for QAT_5 strain, on *Amaranthus retroflexus* (Fig. 2). Greenhouse plants had Subsequent necrosis than plants that were cultured in ms media (This may be due to the

thickness of the leaf cuticle and physical resistance in the greenhouse than MS media, because sometimes physical strength leaf acts as a barrier against pathogens). G_{7-1} on *Rumes crispus* were lead to death 10 days after spraying that were cultured in pots (Fig. 3). While no sign of growth cessation was observed in control treatment, also no symptom was observed on wheat.

Spores don't sediment easily in fungal samples by centrifuge, unlike the bacterial samples, therefore, they should be eliminated using 0.22 µl filter in final step [37]. In this research, the fine fabric silk was also used followed by centrifugation and filtration. In this method a completely uniform supernatant was obtained [38].

In this study the spraying method was used since in this method comparison with other methods the protein is more quickly and homogenously absorbed, due to their homogenous dispersion on the surface of leaves. In addition this procedure has severe damage [28].

Furthermore, the leaf experiences an infinitesimal mechanical stress, due to the small spraying force. Therefore the method was considered to be appropriate.

The protein concentrations of supernatant were 0.1 mg/ml for QAT_5. In more articles, the 24 to 26 kDa protein was reported the cause of necrosis property [34, 39]. In this study, the band around 24 kDa was observed on the SDS-PAGE gel, just for QAT_5 strain, consequently, it was found that this strain is able to eliminate the plant due to the production of desired protein (Fig. 4)

But about G_{7-1} strain there was no band on SDS-page gel even when the protein was concentrated by using ammonium sulfate method, which show this phytotoxine was not a protein (Fig.5).

Basic and hydrophobic properties of the 24kDa protein are similar to those of a subgroup of fungal elicitors including xylanase from *Trichoderma viridae* [41] and elicitin from Phytophtora species [40]. It has not yet been clear whether such similarities influence the protein-plant cell interactions or they are simply a natural property which is common among extracellular proteins [34].

In FPLC method for isolating phytotoxine from QAT_5 strain using cationic column, all negatively charged proteins came out of the cationic column according to ionic strength until 7.30 min, then, positively charged proteins came out of the column using B buffer. Based on previous studies, protein came out of column using B buffer and showed peaks at 10 to 13.3 min, which includes positively charged proteins such as our desired protein, and was collected by the collector. The purification result was obtained using hydrophobic column and based on previous studies, desired protein revealed a peak at about 14 min; therefore, this peak was collected for next step (gel filtration column). The results of protein isolation based on the size showed a peak around 23 min which is related to the desired purified protein.

Heretofore, the purification has been cited in all articles to Bailey method [34]. In this study, the gradual changes of buffer concentration were used instead of step-by-step increasing in buffer concentration.

Figure 1. Phytotoxine effect of QAT_5 on *Amaranthus retroflexus* in greenhouse 1. sprayed plant (A:control, B: effect of spraying) 2. 3 days after spraying 3. 6 days after spraying (A: control, B: effect of spraying).

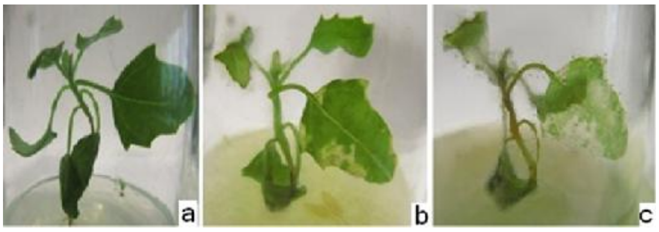

Figure2. Control and evaluation of the supernatant of QAT5 strain, on *Amaranthus retroflexus*. a: sprayed plant, b:3days after spraying, c: 5 days after spraying.

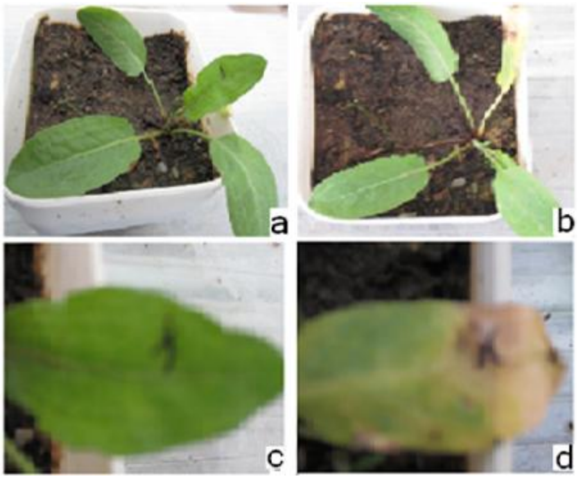

Figure3. Control and evaluation of the supernatant of G7-1 strain, on *Rumes crispus*. a: sprayed plant, b: 3 days after spraying, c: Close location of the spray, d: 10 days after spraying.

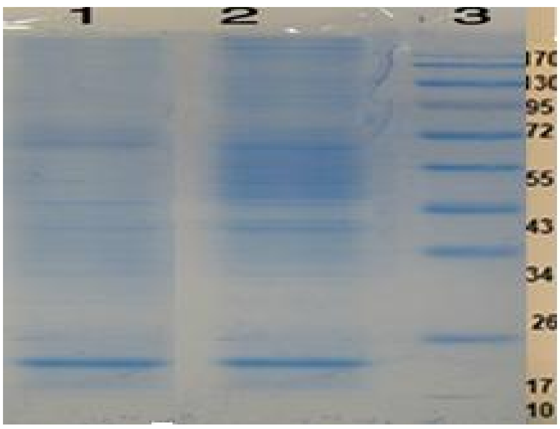

Figure 4. Column 1 and 2, QAT5 strain; Supernatant and concentrated (the concentrated protein solution using ammonium sulfate for better result) respectively on SDS-PAGE, Column 3: weight marker protein

Discussion

One main reason of influencing the decline in crop yield is weeds and no management of them. The widespread use of chemical compounds to control weeds and increase public awareness about Risks associated with these compounds on public health and the environment Problems because they threatening the health of consumers because of pesticide residues in crops, contamination of water, soil and air, The loss of natural enemies, increasing resistance to pests, Necessity to utilize other pest control methods such as biological control has revealed [42,43] Among The comprehensive and integrated approach to weed management, weed control and the use of plants micro organisms, in agricultural ecosystems as an applied factor has been accepted [44]. Among the various microorganisms for biological control, use of pathogenic fungi more applicated. In particular pathogenic fungi plants of necrosis factor, that with synthesizing of a large number of host-specific toxins specific or nonspecific, which facilitate dead tissue, are most interested [45]. NLP protein was isolated from *Fusarium oxysporum* for the first time [34]. Gijzen and Nurnberger in 2006 expressed the properties of this protein, which belongs to NLP family, as follows; they only are active for on dicotyledon, they show an unstable necrosis activity against heat, these proteins act extracellulary, the presence of an elicitor receptor on cell wall was finally recommended which causes the prompt response of immune cells and eventually lead to necrosis and death. Since these phytotoxine according to the result had such a similarity to what founded before they are recommended as biocontrol factor of these weeds instead of chemical herbicides. Also for increasing and having death in rapid time using of these phytotoxine in more time in day unit or using another companion factor like enzymes, detergents are suggested.

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