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A Peptide Extract of Hydrolyzed Amaranth Globulin Induces Growth and Immunological Response in Tomato and Maize Plants

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MFJS and SGM designed the study performed, wrote the protocol, managed the literature searches and wrote the first draft and final of the manuscript. Authors MFJS and RLFJ carried out the experimental section. Author SGM managed to get financial funds for this research project. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Evaluate the effect in growth and defense activation of a peptide extract from hydrolyzed globulin of Amaranth in tomato and maize plants.
Study Design: Using different concentration of peptides we evaluated the physiological effect of peptide extract from amaranth in two different commercial crops.
Place and Duration of Study: Department of Biomacromolecules Chemistry at the Chemistry Institute UNAM, and Plant Pathology Lab, Escuela Nacional de Ciencias Biológicas, UNAM, duration 1 year.
Methodology: For evaluating the effect of peptide extract from globulin (GPE), tomato seeds were geminated in different concentration of GPE (10, 1 and 0.1 mg/mL), 5 seed per treatment were

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used, the effect of germination was observed for the length of epicotyl and stem development. Activation of plant innate immune response was tested using tomato leaf disc system (5 discs per treatment), tomato disc were exposed to 3 concentration of GPE, boiled bacteria *P. syrinage* pv. tomato and plant defense activator BTH for 15 minutes and analyzed with histochemistry detection for ROS. ROS were also quantified in laves of tomato plants treated with GPE, boiled bacteria and BTH, ROS were detected with DMPO using electronic paramagnetic resonance. To evaluate the protective effect in other plants, leaf blight system with *Helminthosporium* sp. was used to know the effect of GPE against fungi infection.

Results: GPE showed an improvement of seedling development at the concentration of 1 mg/mL, while innate immune response was induced after 15 minutes with the concentration of 1 and 0.1 mg/mL of GPE. This result also matched with the observed protection assay in a model infection system of leaf blight, showing protection of several blight development at 0.1 mg/mL.

Conclusions: The peptides derived from hydrolyzed globulin of *Amaranthus hypochondriacus* induce promotion in growth and develop of tomato, also innate defense in tomato leaves and in maize against fungi infections.

Keywords: Amaranthus hypochondriacus; peptides; hydrolyzed globulin; induce promotion; growth and develop; tomato; maize; fungi infections.

1. INTRODUCTION

Amaranthus hypochondriacus is a Mexican plant that have been used since prehispanic times, the amaranth seed contains a large content of proteins compared with other Mexicans grain plants like maize, chia [1,2,3]. The total content of proteins is around 20%, globulins and albumins, represent approximately 48 and 20% respectively, in the total protein fraction [4,5]. Several reports described the nutraceutical activities of the amaranth proteins, and the derived digestion products of these proteins [6]. The main activity of amaranth protein sub-products is the anti-hypertensive function. the amaranth proteins are used mainly for nutraceutical approach, there are no reports about the use of amaranth proteins and sub-products in agricultural applications [6,7]. Peptides have showed to be an attractive crop protection agent in plant systems [8] inducing plant immunity [9], growth promotion [10], anti-viral [11], and anti-bacterial bioactivity [12], however, the synthesis of synthetic peptides entails high production cost. Transgenic plants overexpressing peptides are a good alternative for crop protection; nevertheless, the regulation of genetically modified organism has not a good opinion between populations, yet. Cereal and pseudo-cereal plants have a larger amount of proteins in the seeds comparing with other plant species, these proteins are able to be extracted in easy way with high yield extraction and also represent a good source of obtaining peptides with different bioactivities after digestion with enzymes. The variability in size and physicochemical properties of peptides can increase the potential bioactivity [13]. In this work, we explored the potential agricultural application of a peptide extract derived from digested globulin of amaranth (*A. hypochondriacus*), evaluating the effect in germination and development in other to market Mexican tomato plant and also evaluating the capability to induce innate immune response for a potential use in crop protection.

2. MATERIALS AND METHODS

2.1 Geographical Study Area

The geographical localization of this experiment was carried out at University City, National Autonomous University of Mexico, UNAM in México City, longitude: 19.327531, latitude: -99.178831. In Mexico City, the huge capital of Mexico, the climate is subtropical, mild or pleasantly warm during the day, with cool nights in summer and cold nights in winter. In fact, the daily temperature range is remarkable, especially in the dry season with average temperature of 23.5℃.

2.2 Protein Extraction and Hydrolysis

Amarantus hypocondriacus seeds were ground, the powder was filtered in gauze to remove the fiber. The flour was defatted overnight at 4℃ with n-hexane (1:10 w/v) in stirring, then centrifuged at 10000 rpm / 15 min, and the pellets were recovered and dried at room temperature. After that, the flour was suspended in water and the pH was adjusted to 8.5 with

NaOH 0.1 M (modified protocol Romero-Zepeda, H., & Paredes-Lopez) [14], and incubated overnight for extracting the soluble proteins at alkaline pH. The protein extract was then centrifuged at 10000 rpm/ 15 min to delete the starch and non-soluble proteins. The supernatant was recovered, and re adjusted the pH to 7.0 and used for enzymatic digestion. To know the extracted proteins in the supernatant, a SDS-PAGE was performed.

For hydrolysis, one litter of protein extract was mixed with papain (Sigma-Aldrich) in proportion 0.5 g/L and incubated for 15 hours at 37℃ to control the hydrolysis activity avoiding the completely proteolysis of proteins. After the incubation time, peptide extract was fractioned in Amicon pressure system with a membrane of 10 KDa, fraction less than 10 KDa were collected and concentrated with cryo-concentration method [15]. C18 RP-HPLC was used to verify the peptide formation after hydrolysis.

2.3 Effect of Globulins Peptide Extract (GPE) in Germination of Tomato Seeds

Tomato seeds commercial variety “Rio Grande” was supplied by the Universidad Autónoma Chapingo, Mexico, were disinfected with common sodium hypochlorite 3% for 5 minutes, and washing with sterile distillated water 3 times to remove the excess of disinfectant. For germination, we used a mix of vermiculite and commercial black soil for vegetables in a proportion 1:1 (w/w), the mix was autoclaved before use. The germination substrate was soaked in different treatments: 1) Water, 2) Commercial Hydroponic nutritive solution Hydro-sol (5:11:26; N:P:K, respectively), 3) GPE 10 mg/mL, 4) GPE 1 mg/mL and 5)GPE 0.1 mg/mL. Once the substrate was prepared, the seeds were seeded in the different treatment lots, 5 independent seeds per treatment. Then the plant germination system was incubated in dark until first signs of germination (emergence of epicotyl and cotyledon), at this time, the measurement of growth of the plant just started and the germination trays were then changed to a light/dark photoperiod (16 hours light/8 hours darkness). The growth and development of the plants were evaluated for next 15 days. The substrate was watered with testing solutions every 2 days during experimental time. The length of the tail or epicotyl in each treatment was used for comparing the effect between each experimental conditions.

2.4 Induction of Reactive Oxygen Species (ROS) Production in Tomato Leaves Mediated for GPE

Tomato plants with 3 weeks-old were used to evaluate he ROS production mediated for GPE. Full expanded new leaves were used for preparing leaf disk around 10 mm of diameter. The leaf’s discs were floated in sterile distillated water in dark Cconditions overnight, then, four treatments were used for study the ROS production: 1) Distillated water, 2) BTH 300 mg/L, 3) 10 mg/mL of GPE and 1 mg/mL of GPE, GPE solution and BTH were diluted in sterile distillated water, 5 leaf discs per treatment were used to evaluate the immune response, 100 µL of each solution were added on leaf discs for 15 minutes, after they were washed 3 times with sterile distillated water and staining for detection of peroxide and superoxide. Peroxide was detected with Diaminobenzidine (Sigma-Aldrich) and superoxide was detected with nitro tetrazolium blue, NTB (Sigma-Aldrich). The presence of brown precipitated or blue complex in the tissue is indicative of peroxide and superoxide production, respectively.

2.5 ROS Quantification Using Electron Paramagnetic Resonance

In order to quantify the ROS production, we performed electron paramagnetic resonance (EPR) to quantify the peroxide and superoxide, in brief, we used leaves of the same plants mentioned in the above section, plants leaves were sprayed with the treatments until saturation: 1) Distillated water, 2) BTH 300 mg/L, 3) 10 mg/mL of GPE and 4) 1 mg/mL of GPE and 5) boiled suspension of bacteria Pseudomonas syringe pv. Tomato, D.O.600 nm=0.2, GPE solution, BTH and bacteria were diluted in sterile distillated water. 100 mg of leaf sample of each treatment were sampled at different times after spraying the experimental solutions, the samples were taken and immediately added 10 µL of DMPO (Sigma), and then ground fast in tissue lysis machine for 1 minute, after that, the ground material was transferred in a EPR cell. The sample was read in the EPR machine, Jeol JES TE-300 Xband, 100 KHz, and Modulation. The intensity of DMPO adducts coupled with the ROS radical was used for relative quantification of ROS production. The intensity signal of negative control (plants treated with water) was used for comparing the significant difference between treatments; Two-Way ANOVA was used for

comparing treatments between different groups. Data were analyzed in GraphPad Prism 6.0.

2.6 Protection Assay against Leaf Blight in Maize

A four week old new leaf of *Zea maize* from 5 different plants were used for experiment, 3 different leaf segments with 4 treated spots per leaf segment were used for each experimental condition. The experimental conditions were: Healthy control (treated with sterile water), infected control (treated with sterile water and infected with suspension of 120000 conidia/mL of *Helminthosporium* sp, BTH 300 mg/L infected with suspension of 120000 conidia/mL, GPE 1 mg/mL infected with suspension of 120000 conidia/mL and PGE 0.1 mg/mL with suspension of 120000 conidia/mL . For treatment, 50 µL of sterile water for healthy and infected control, and 50 uL of BTH and GPE solution all dissolved in sterile water were dropped in 4 equidistant points in each leaf segments, the testing solutions were allowed to absorb on the leaf for 2 hours. After this time, 10 µL of conidia suspension of *Helminsthosporium* sp. was inoculated in the treated spot. Leaves were incubated in humidity chamber, under photoperiod 16 hours light/8 hours darkness. The symptoms develop or hypersensitive reaction was monitored for 5 days.

3. RESULTS AND DISCUSSION

3.1 GPE Stimulates Growth in Tomato Seedlings

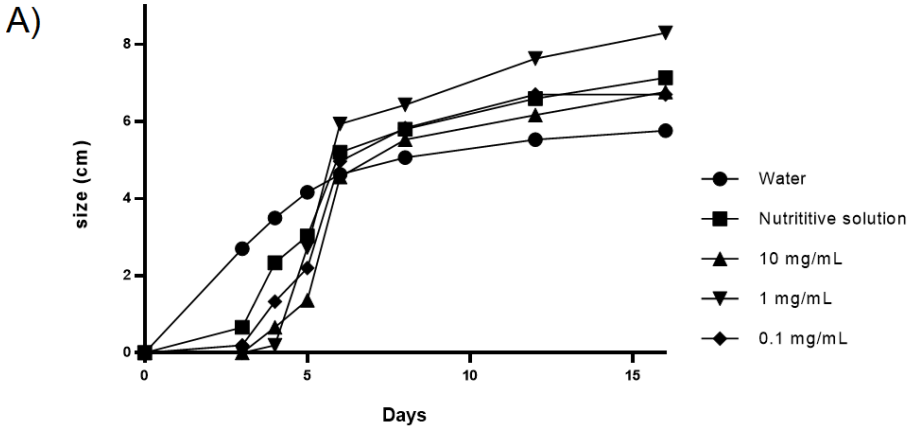
Seeds germinated in presence of GPE improves development of tomato seedlings inducing the increase in size, leaf development and size compared to seeds germinated in traditional water system (Fig. 1) It has been demonstrated that the commercial formulation "Trainer" that is composed of a vegetable protein digest, induces the rooting in tomato, as well as its elongation of the stem. Likewise, this commercial formulation induces elongation of coleoptile in maize, similar to the effect of some phytohormones, such as auxins [16]. In this work, it is possible to observe a correlated effect exerted by the GPE extract on tomato seedlings in the initial stages of development and that is also reflected until stages of foliar proliferation. At concentrations of 1 mg/mL of GPE, which is a lower concentration than that used with “Trainer”, the size of the plant increases more than plants treated with nutritive solution, an excess of peptide 10 mg/mL does not show a significant effect in growing compared with nutritive solution, but is notable compared to the treatments that were just treated with water (Fig. 1). The excess of peptide could be inducing intermolecular interaction between peptide that disturbs the function in the cell, the same effect was observed with maize seedlings treated with “Trainer”, high concentrations of peptide digest product decreases the bio stimulating effect.

3.2 GPE Induces Activation of Innate Plant Response in Tomato Plants

Several peptides derived of pathogen related proteins have been reported for be pathogen associated molecular patterns (PAMPs) to activate the innate defense response in plants. These peptides are short sequence, less than 10 KDa, for example the pathogen associated peptide flg22 and some peptides included in the family PROPEP, the initial response that these peptide produce is the induction of ROS production in apoplastic space [17], to generate an oxidative microenvironment to control the pathogens, with subsequent strong and more specific immunological pathways activation. This work shows that peptides extract from GPE can activate the ROS production in tomato leaves, specifically peroxide and superoxide, in Fig. 2a, tomato leaves produce peroxide (brown precipitate) and superoxide (blue precipitate) in apoplastic regions. That activity was also verified in a different plant, we used the model system plant *Arabidopsis thaliana* Col-0 to extrapolate the results to other species. *A. thaliana* also shows the production of superoxide (blue colored) after GPE exposition for 15 minutes (Fig. 2b). That response in both plants that agree with the time reported for ROS production after peptide like PAMP induction [9,17].

To quantify the oxygen radicals production, EPR was used, the results in Fig. 2c shows a significant increase of ROS response in plants treated with 0.1 mg/mL, the response of ROS production is even greater than that generated in plants treated with a boiled bacteria culture of *P. syringae* pv *tomato*. The response in ROS production shows a decrease after 2 hours, this result is indicating that ROS production is like those with tomato leaf discs in other experiment with tomato. In this experiment is included one commercial immune inductor, that is analogue of salicylic acid, this was acibenzolar-S-methyl, BTH (Actigard, Syngenta), this molecule as same salicylic acid, reduce the oxidative burst in the

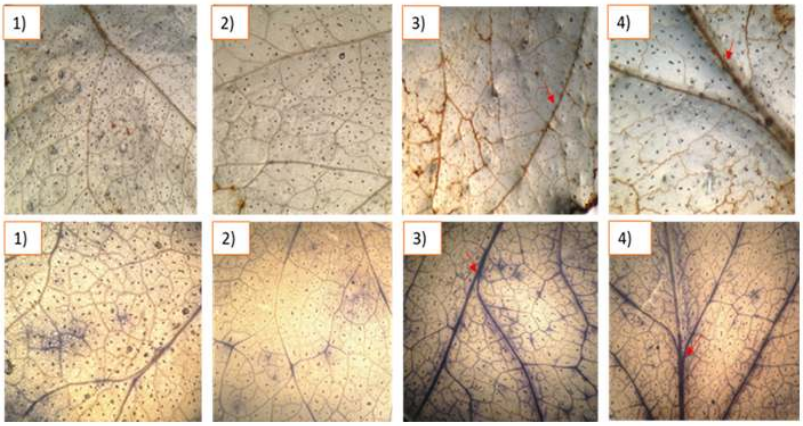
first step of plant immune activation but make also after time can induce a lightly ROS production for increase the SA synthesis in the plant to increase the immune response, the results showed no significant difference between water treated plant and plant treated with BTH in the first 15 minutes, however after 1.5 hour, the feedback response of ROS production increase lightly significant.



B)

Fig. 1. Amaranth GPE induces growth promotion in tomato seedlings. A) Effect of GPE on tomato seed germination under different solutions: Water, Nutritive solution Hydrosol ®, GPE 10, 1 and 0.1 mg/mL, GPE at 1 mg/mL. B) Phenotypic effect of GPE in tomato "Rio Grande" at 15 days post germination in different conditions in substrate, substrated soaked with 1) water, 2) nutritive solution Hydrosol ®, 3) GPE 10 mg/mL, 4) 1 mg/mL and 5) 0.1 mg/mL

a)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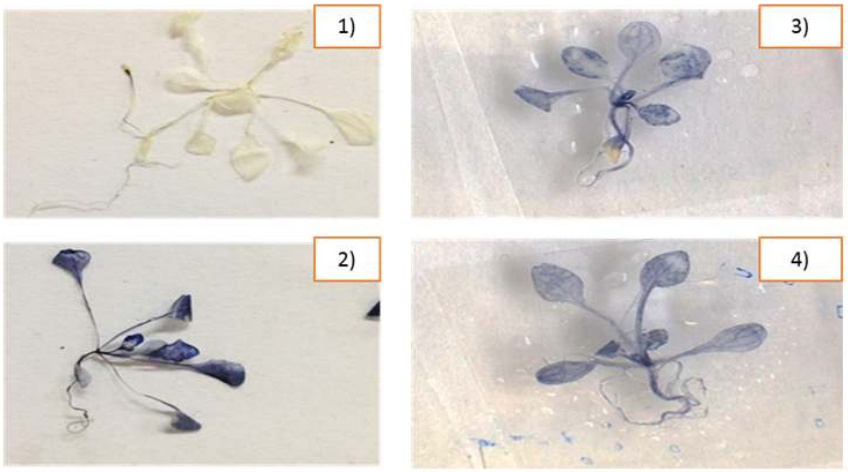
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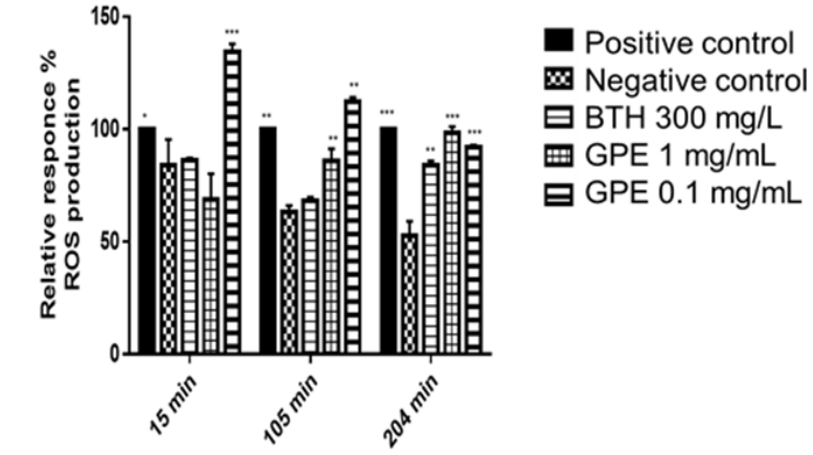
Relative responce % ROS production
150
100
50
0
15 min
105 min
204 min
Positive control
Negative control
BTH 300 mg/L
GPE 1 mg/mL
GPE 0.1 mg/mL

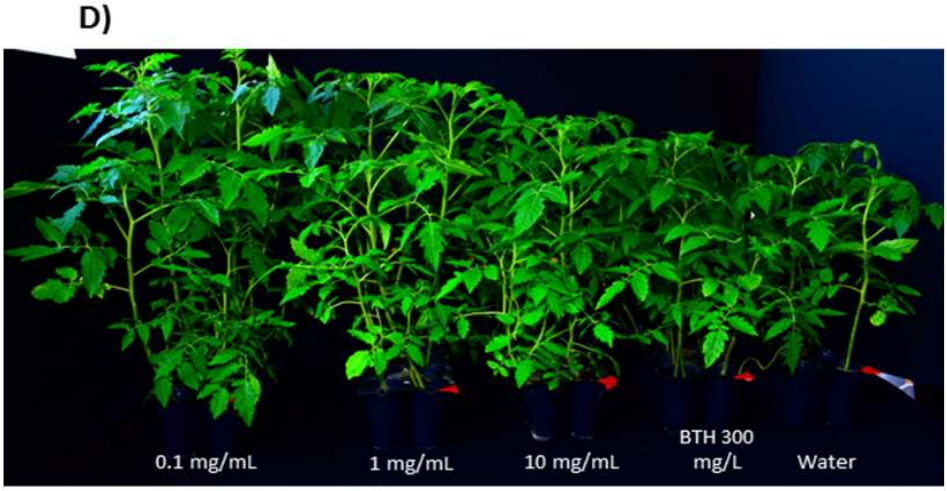

Fig. 2. Activation of immune pathway in *Arabidpsis thaliana* col-0 and Tomato leaves with GPE. A) The innate immune response activation in tomato plant. ROS like H_2O_2 (upper B panel) and O-2 (lower B panel) production was evaluated using Diaminobenzidine and NTB, respectively. 1) Distillated water, 2) BTH 300 mg/L, 3) 10 mg/mL of GPE in water and 4) 1 mg/mL of GPE in water. H_2O_2 and O-2 production show a brown and blue precipitation, respectively. B) ROS production in *A. thalaiana* col-0 seedlings with GPE, B.1) Distillated water, B.2) boiled bacteria suspension D.O 0.2, B.3) 1 mg/mL of GPE in water and B.4) 10 mg/mL of GPE in water. C) ROS quantification with Electronic paramagnetic resonance (EPR) in tomato leaves. The relative production response was normalized using the positive control like 100% of ROS signal in EPR. Error bars represent SD, asterisk indicate a significant difference according (P = 0.05). D) Phenotypic effect of GPE in tomato plants od 5 old-weeks, the GPE induce growth promotion comparing with other plant activator like BTH

Plants treated with peptide and salicylic acid showed difference in phenotype after 15 days post treatment, salicylic acid and analogues are well known that are antagonist as some growth hormones, reducing size of plants, but at the same time inducing protection, this work shows that PGE induce related- innate defense molecules and also promotes plant growth, Fig. 2a, d.

3.3 GPE Induces Protection in Maize against Leaf Blight

The activation of immune response showed in tomato was also explored in Maize, with an infection model of leaf blight caused by *Helminthosporium* sp. This fungi strain was isolated from maize cultivation field in Tenango City, Estado de México, México, and characterized in research group of Professor Luna-Romero. GPE extract at concentration of 1 and 0.1 mg/ml induced protection of classic leaf blight development (Fig. 3) compared with the infected control pre-treated with water, these results matched with those observed in tomato plants for ROS production, supporting our idea of protein extract might contain some peptides with similar sequence of pathogen peptide like-PAMP with capability to induce innate immune response, avoiding pathogen proliferation. However, unlike those MAMP-like peptides such as flg22, AtPep1 and their Pep1 / 2 homologs, the peptides present in GPE not only induce the appearance of molecules associated with immunity downstream of the MAMP recognition, but, in tomato plants, these peptides also showed a favorable effect on growth [18].

In *A.thaliana*, when one of the receptors for flg22 or AtPep1 has been mutated, the antagonistic effect of growth is slightly reversed [19]. In the findings reported in this work, it has shown that the GPE peptide extract induces not only immunological activation but also promotes growth, meaning that the peptides present in the extract may be partially recognized as MAMP or peptides associated with damage (DAMP) but not recognized by LRR-K receptors with high affinity or specificity as flg22 or Pep1 / 2 do [19,20].

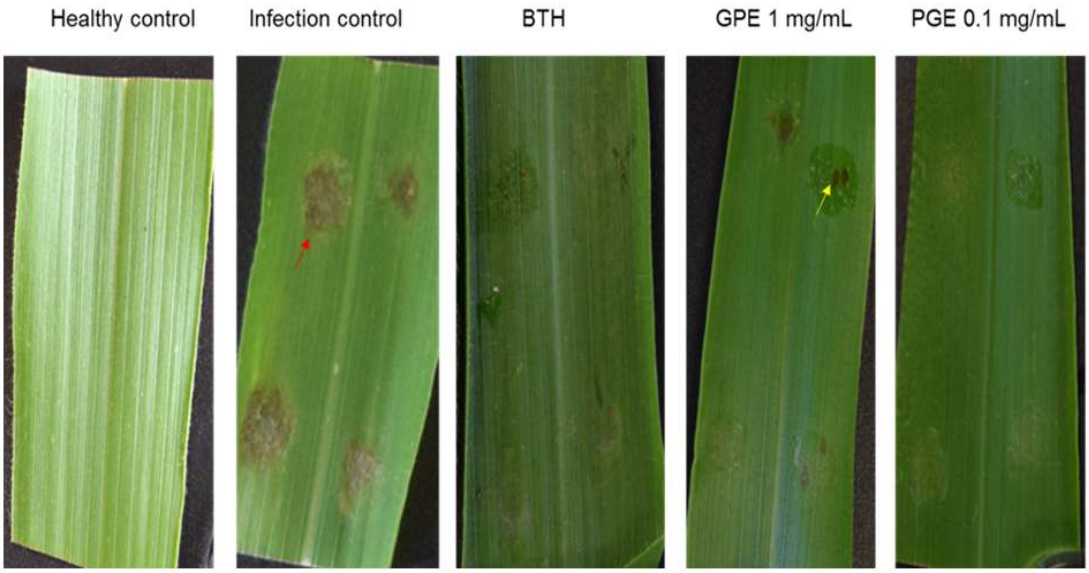

Fig. 3. GPE of amaranth induce protection in maize against leaf blight. Comparing GPE treatments versus infection control they decrease the characteristic leaf blight symptom (red arrow). Concentration on 1 mg/mL of GPE induces small response of hypersensitivity (white arrow) since one day after treatment

It has been reported that some peptides of Glycine max from its precursor PROPEP, induce defense in these seedlings and growth induction after an exogenous application, activating genes for the synthesis of a nucleotide-binding site leucine-rich repeat protein (NBS-LRR), pectin methylesterase inhibitor (PMEI), Respiratory Burst Oxidase Protein D (RBOHD), indicating a transcriptional reprogramming, which generates not only defense, but also reprogramming in developmental genes [21]. The main possible mechanism of action of peptides presents in GPE could be homologues to this last argument. Analysis of peptide sequences presents in performing to know the similarity of GPE peptides with peptides from PROPEP precursors.

4. CONCLUSION

The extract of peptides derived from the enzymatic digestion of amaranth globulins has a bio-stimulating effect in tomato plants by inducing growth and increase development in leaves at concentrations of 1 and 0.1 mg / mL, also this extract induces the production of immunological molecules such as ROS in tomato leaves. In maize the peptide extract reduces symptoms of blight caused by *Helminthosporium* sp.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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