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Research Article
Growth Promotion and Disease Resistance in Muskmelon Induced by Crude Proteins of *Penicillium verruculosum* Against Gummy Stem Blight Disease

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Abstract

Background and Objective: Melon cultivation is more depended on synthetic fungicides to control gummy stem blight caused by *Stagonosporopsis cucurbitacearum* (KJ782214). This study mainly aimed to minimize the use of harmful chemicals to control disease by inventing the biological alternatives. **Materials and Methods:** The crude proteins (CP) were extracted from rhizospheric fungi *Penicillium verruculosum* (*Talaromyces verruculosus*) (KU645999) to understand the growth promoting and disease protection ability of CP at four concentrations against gummy stem blight pathogen. **Results:** The CP treatment at 100 µg mL^{-1} for 6 h showed enhance vegetative growth parameters like germination (90%), seedling vigor (2329.11), plant height (42.35 cm), number of leaves (5) and root and shoot weights (0.05-2.3 g) and chlorophyll content (4.16 mg g^{-1}) when compared to control. The CP treatment at 100 µg mL^{-1} for 6 h also exhibits considerable disease protection of 74.37%, which was found to be higher compared to control and almost equal to the effectiveness of fungicide (Mancozeb) treated (94.03%) plants. **Conclusion:** Present study concludes with the invention of protein composition of the rhizospheric fungi from muskmelon field as potential alternative to harmful chemicals in controlling the disease.

Key words: *Cucumis melo*, gummy stem blight, *Stagonosporopsis cucurbitacearum*, plant growth-promoting fungi, crude protein elicitors, disease protection

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Plants protect themselves against broad range of pathogens by developing complex immune system[1,2]. The immune system is triggered by the mediators, such chemicals are known as "elicitors". The term elicitor was used for molecules capable of inducing the production of active compounds. They do not have any common chemical structure. These chemicals belong to a wide range of different classes of compounds viz., oligosaccharides, peptides, proteins, glycoproteins and sphingolipids[3]. Many elicitors have been isolated from various organisms, viz., bacteria, viruses and fungi to improve the pathogen resistance of plants. With the discovery of protein elicitors, there has been great interest in manipulating the inducible responses of plants for crop protection. Searching for novel protein elicitors has become a popular strategy in plant disease control[4,1]. A bio-preparation has been used successfully in many plants to elicit plant disease and insect resistance, to promote growth, yield and quality[5]. Many protein elicitors have been isolated and used to improve the pathogen resistance of plants. For example, protein elicitor harpins were first isolated from *Erwinia amylovora*[6]. They are glycine-rich proteins that lack cysteine, show heat-stable HR elicitor activity when infiltrated into the leaves of tobacco and several other plants[7-8]. It was shown to elicit the hypersensitive reaction (HR) and induce disease resistance in many plants[6,9-12].

Gummy stem blight (GSB) disease is caused by *Stagonosporopsis cucurbitacearum* (Fr.:Fr.) Aveskamp, Gruyter and Verkley, which leads to severe yield losses in melons (*Cucumis melo* L.), watermelons (*Citrullus vulgaris* Schrad.), cucumbers (*Cucumis sativus* L.) and other Cucurbitaceae crops[13]. The GSB was observed and identified out in the early 1980s in Europe[14,15], in the United States[16-18], China, Japan and other tropical and subtropical countries[19,20]. The fungus causes seedling damping-off, foliar lesions as well as, stalk and stems cankers. It can be universally found in every continent and attacks at least 12 genera and 23 species of cucurbit plants[21]. Farmers depend mainly on synthetic fungicides to control cucurbit diseases. However, in addition to environmental pollution and contamination of consumable products, several cases of resistance to the main active fungicides on the market have been reported for GSB[22,23]. There is a need for alternative eco-friendly biological control measures. Hence, the aim of the present study was to investigate the effect of crude proteins from rhizospheric fungi on the plant growth and induction of disease protection in muskmelon against gummy stem blight disease. For this purpose, proteins were extracted from the rhizospheric fungi *Penicillium verruculosum* (the revised name: *Talaromyces verruculosus*) which, gave very good results of growth promotion and disease protection when primed to the seeds treated with conidial suspension in our previous studies.

MATERIALS AND METHODS

Collection of muskmelon infected plants and seed samples: Field survey was conducted in muskmelon growing agroclimatic zones of Karnataka for the collection of infected plant samples. Muskmelon plants were diagnosed on the basis of typical symptoms of GSB like, stem necrosis with gummy exudate, angular water-soaked lesions on the leaves. Seed samples were collected from the farmers, agricultural farms, Public and Private Seed Agencies, brought to the laboratory and stored at 4°C for further use.

Screening and isolation of *S. cucurbitacearum*: Seeds from infected fruits and symptomatic plant parts collected from fields of muskmelon were surface sterilized with 0.2% sodium hypochlorite (NaOCl) for 5 min and repeatedly washed with Sterile Distilled Water (SDW) to remove traces of sterilant. These samples were screened for pathogen following Standard Blotter Method (SBM) and incubated for 7 days at 25±2°C [24]. After the incubation period, each sample was examined under Stereomicroscope for fungal colonies showing typical sporulating structure of *S. cucurbitacearum.* These colonies were aseptically picked with sterile needle and transferred on to Potato Dextrose Agar (PDA) medium. Pathogen was identified based on morphological, conidial, cultural characteristics[25], sequence analysis of ITS region and comparison analysis with GenBank data. Sequence was deposited in GenBank and obtained the accession number (KJ782214).

Isolation and identification of rhizospheric fungi: One gram of soil sample was suspended in 9 mL of SDW. An aliquot of 0.1 mL of 10^{-3} to 10^{-5} dilutions from each of the soil samples were prepared. About 1 mL of each soil dilution was inoculated on PDA plates supplemented with antibiotic (100 mg L^{-1}) under aseptic condition and incubated for 7 days at 25±2°C. The fungus was identified as *Penicillium verruculosum* (MRS-PGPF 24) on the basis of microscopic (mycelia and conidia) and macroscopic (culture morphology and appearance) characteristics[25], sequence analysis of ITS region and comparison analysis with GenBank data. Sequence was deposited in GenBank and obtained the accession number (KU645999).

Preparation of pathogen (*S. cucurbitacearum*) inoculum: Spore suspension of *S. cucurbitacearum* was prepared using SDW by harvesting spores by lightly scraping the surface of the sporulating PDA culture plates with a sterilized spatula under aseptic conditions. The concentration of the inoculum was adjusted to 1×10^5 spores/mL using Haemocytometer[26] and used for further studies.

Mass production of *Penicillium verruculosum* (MRS-PGPF 24) for elicitor extraction: Fungi that stood best for growth promotion and disease protection was subjected for extraction of crude elicitors (Proteins). Fungal colonies were picked from the actively growing margin of the PDA plates (5 mm) with a sterile fine-tipped needle and transferred on to 500 mL Erlenmeyer flask containing 200 mL PDB. The inoculated PDB was incubated at 25±2°C under 12/12 h alternate cycles of light and darkness for 15 days. Fungal mat was separated from crude culture filtrate by pouring through two layers of cheese cloth. This was further filtered through two layers of Whatman No.2 filter paper[27].

Extraction of cell wall crude proteins (CP) from *Penicillium verruculosum* (MRS-PGPF 24): Crude protein extract was isolated by the modified method of Djonovic *et al.*[28]. The fungal liquid culture was strained through two layers of cheese cloth. Each of the fungal supernatant was subjected to $(NH)_4SO_4$ precipitation for 5 h at 4°C with continuous stirring while monitoring the pH (pH 7). The pellet obtained at 80% saturation was collected by centrifugation at 12,000 g for 15 min at 4°C and dissolved in minimal volume of column buffer (50 mM sodium phosphate, 0.15 M NaCl, pH 7.0). Samples were desalted by applying them on to desalting column (Genei) to remove the ammonium sulphate. The amount of protein was estimated using a Bradford protein assay with bovine serum albumin (BSA) as standard. The CP powder was diluted with SDW to prepare various concentrations (50, 100, 150 and 200 μg mL^{-1}) and used for seed treatment.

Muskmelon seed treatment with crude proteins (CP) from *Penicillium verruculosum* (MRS-PGPF 24): Seeds of muskmelon were surface sterilized with 0.2% NaOCl for 5 min, followed by repeated washing with SDW. After surface sterilization the seed samples were treated with CP at 50, 100, 150 and 200 μg mL^{-1} for 3 and 6 h, respectively. After the incubation period treated seeds were air dried aseptically and further used to study their efficacy of muskmelon plant growth promotion as well as protection against gummy stem blight disease. Distilled water treated seeds and fungicide (Mancozeb) treated seeds served as negative and positive control, respectively.

Effect of seed treatment with Crude Proteins (CP) from *Penicillium verruculosum* (MRS-PGPF 24) on seed germination and seedling vigor of muskmelon under *in vitro* conditions: The seed treatments with CP and SDW were used to study the growth of muskmelon.

The seeds were evaluated for germination (%)[29] and seedling vigor[30]. The experiment consisted of four replicates of 100 seeds each and was repeated thrice. After 7 days of incubation at 25±2°C germination (%) and vigor index was calculated as mentioned below:

$$\text{Germination (\%)} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds plated}} \times 100$$

$$\text{Vigor index} = \text{Seed germination (\%)} \times [\text{Mean Root Length} + \text{Mean Shoot Length}]$$

Effect of seed treatment with Crude Proteins (CP) from *Penicillium verruculosum* (MRS-PGPF 24) on vegetative growth parameters of muskmelon under green house conditions: The CP (50, 100, 150 and 200 μg mL^{-1}) and SDW treated susceptible seeds were sown in pots containing soil, sand and farmyard manure (FYM) in the ratio 2:1:1. Total 4 seeds were sowed to each pot and watered regularly up to 30 days. Growth promotion was evaluated under green house conditions by recording root length, shoot length, plant height, root and shoot fresh weight and dry weight, number of leaves per plant and chlorophyll content according to the method of Hiscox and Israelstam[31]. The experiment was repeated thrice with four replicates of each treatment. Total chlorophyll in the sample was calculated using the following formula:

$$\text{Total chlorophyll} = [20.0\ (A_{645}) + 8.02(A_{663})] \times [V/100 \times W \times a]$$

Where:

A : Absorbance at specific wave length (645 and 663 nm)
V : Final volume of the chlorophyll extract (mL)
W : Fresh weight of the sample (g)
a : Path length of light (cm)

Effect of seed treatment with Crude Proteins (CP) from Penicillium verruculosum (MRS-PGPF 24) on muskmelon gummy stem blight disease protection under green house conditions: Plants raised from seeds primed with CP were

challenge inoculated with pathogen after first true leaf emerges (15 days old). Disease (%) was recorded periodically (every 7 days) by observing the number of infected leaves[26]. The seedlings raised from SDW treated seeds and fungicide treated seeds served as negative and positive control respectively. The experiment was repeated thrice with four replicates of each treatment. Disease protection (DP) of *S. cucurbitacearum* was calculated using the formula:

$$\text{Disease protection}\,(\%) = \frac{C - T}{C} \times 100$$

where, C represents disease (%) incidence in control, T represents disease (%) incidence in treated plants.

Statistical analysis: Each experimental data was subjected to analysis of variance (one way ANOVA) using SPSS Inc., 16.0. Significant effects of treatments were determined by the magnitude of the F-value ($p \leq 0.05$). Treatment means were separated by Tukey's HSD test.

RESULTS

Effect of seed treatment with Crude Proteins (CP) from PGPF (MRS-PGPF 24) on seed germination and seedling vigor of muskmelon under *in vitro* conditions: Crude protein elicitors were tested for their effect on seed quality parameters in different concentrations and time intervals as explained in methodology. The seeds treated with elicitor recorded higher seed germination and seedling vigor when compared to control set. However, the enhancement of seed germination (%) and seedling vigor varied with different concentrations tested at two time intervals 3 h (Fig. 1a) and 6 h (Fig. 1b). Among them CP at 100 µg mL^{-1} concentration recorded highest seed germination of 90% and 2329.11 seedling vigor, followed by 150 µg mL^{-1} with 86.25% seed germination, 2242.23 seedling vigor at 6 h treatment (Fig. 1b).

Effect of seed treatment with Crude Proteins (CP) from PGPF (MRS-PGPF 24) on vegetative growth parameters of muskmelon under green house conditions: All the four CP elicitor concentrations were tested for enhanced growth parameters under green house conditions. The vegetative growth parameters such as plant height, number of leaves, shoot fresh and dry weight, root fresh and dry weight and total chlorophyll content were significantly enhanced on

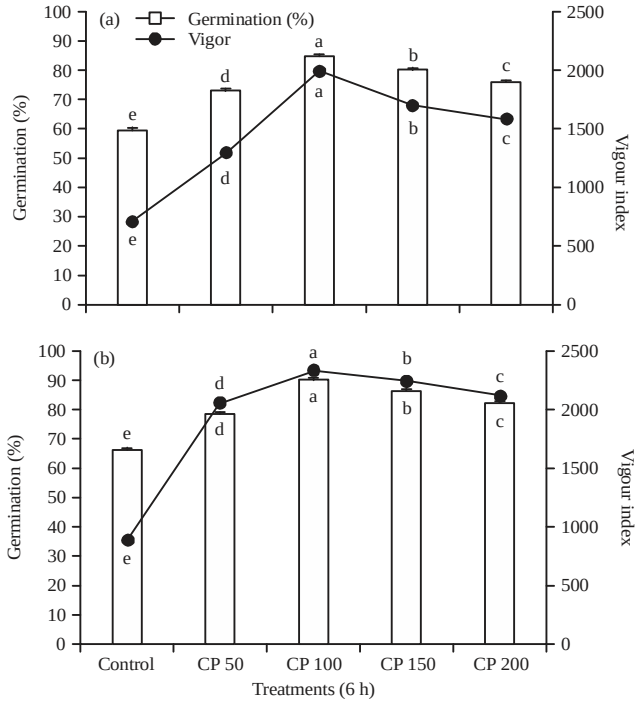

Fig. 1(a-b): Effect of seed treatment with crude protein elicitors on gummy stem blight disease protection of muskmelon under green house conditions. (a) Crude protein treatment at 3 h, (b) Crude protein treatment at 6 h.

Values are means of four independent replicates. Vertical bars indicate ± SE. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD

treatment with elicitors at the end of the experimental period. However, the level of growth promotion varied with the concentration of elicitors. Among the elicitor treatments, CP at 100 µg mL^{-1} concentration treated seeds recorded highest vegetative growth parameters with 42.35 cm plant height, 5 number of leaves, shoot fresh weight of 2.3 g, shoot dry weight of 1.30 g, root fresh weight of 0.43 g, root dry weight of 0.05 g and chlorophyll content of 4.16 mg g^{-1}. The SDW treated seeds showed 21 cm height, 3 number of leaves, 0.5 g shoot fresh weight, 0.04 g shoot dry weight, 0.10 g root fresh weight, 0.01 g root dry weight and 0.05 mg g^{-1} chlorophyll content (Table 1).

Effect of seed treatment with Crude Proteins (CP) from PGPF (MRS-PGPF 24) on muskmelon gummy stem blight disease protection under green house conditions: The crude protein elicitor concentrations which offered best results than control for seed germination and seedling vigor was further tested for

Table 1: Effect of crude protein elicitors on growth of muskmelon seedlings under green house conditions at 6 h

Treatments	Plant height (c)	Number of leaves	S Fresh weight (g)	S Dry weight (g)	R fresh weight (%)	R Dry weight (%)	Chlorophyll (mg g^{-1})
Control	21.00±0.00^{e}	3±0^{c}	0.5±0^{e}	0.04±0^{d}	0.10±0^{d}	0.01±0^{c}	0.05±0.0085^{e}
CP 50 (in µg)	38.60±0.11^{d}	4±0^{b}	1.7±0^{d}	0.15±0.0054^{c}	0.30±0^{c}	0.04±0^{b}	1.90±0^{d}
CP 100 (in µg)	42.35±0.13^{a}	5±0^{a}	2.3±0^{a}	1.30±0^{a}	0.43±0.0075^{a}	0.05±0^{a}	4.16±0.0202^{a}
CP 150 (in µg)	39.75±0.06^{c}	4±0^{b}	2.0±0^{b}	1.00±0^{b}	0.40±0^{b}	0.05±0^{a}	3.12±0^{b}
CP 200 (in µg)	40.98±0.13^{b}	4±0^{b}	1.9±0.0025^{c}	1.00±0^{b}	0.30±0^{c}	0.04±0^{b}	2.69±0.1875^{c}

Values are means of four independent replicates. ±Indicate standard errors. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD

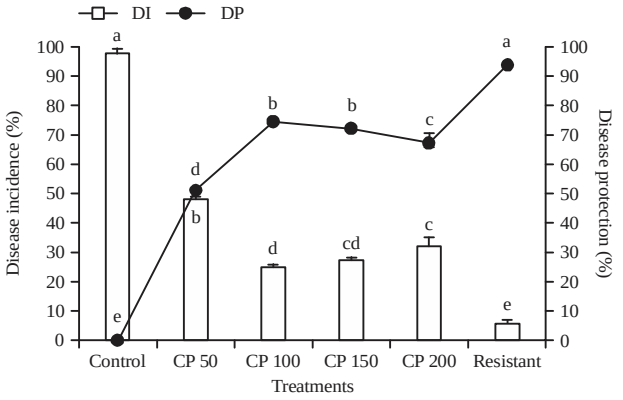

Fig. 2: Effect of Seed treatment with crude protein elicitors on gummy stem blight disease protection of muskmelon under greenhouse conditions.

Values are means of four independent replicates. Vertical bars indicate±SE. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD

its efficacy to induce resistance in muskmelon against gummy stem blight disease under green house conditions. Here resistant treatment with Mancozeb fungicide was used as positive control. It was observed that fungicide treatment offered a maximum of 94.03% disease protection, whereas CP at 100 µg mL^{-1} concentration recorded 74.49% disease protection (Fig. 2). Among the four concentrations tested CP at 100 µg mL^{-1} recorded potential results in inducing disease resistance under green house conditions.

DISCUSSION

Rhizospheric fungi are studied for their growth promotion abilities and induction of resistance against wide array of pathogens. There is a continuous antagonistic war between the useful nonpathogenic microbes with the pathogens. This mechanism also holds good when tested directly as the seed inoculum of such beneficial organisms. In the preliminary studies various such beneficial fungi from healthy muskmelon rhizospheric soil were isolated to evaluate and identify their protective nature towards plants health. Among the isolates *P. verruculosum* was found to be very effective in promoting the muskmelon growth and also induction of resistance against gummy stem blight disease. These pathogens and rhizospheric fungal isolates were deposited in GenBank and obtained the accession numbers KJ782214 and KU645999, respectively. This was further selected to identify the compound that is responsible for this beneficial character of the fungus. Similar studies were conducted by various researchers against different host pathogen systems using soil borne fungi and bacteria[32-37].

Elicitors are molecules that induces some biochemical defense responses during the infection by pathogens in resistant hosts. These molecules are studied extensively to understand the resistance mechanism in the host plants and also to induce resistance against array of disease causing organisms in plants[3,38,37]. In the present study crude protein elicitors from the culture filtrate of *P. verruculosum* seeds were primed with four concentrations (50, 100, 150 and 200 µg mL^{-1}), tested for growth promotion activities and resistance induction in muskmelon against gummy stem blight disease. Similar studies conducted by Wu *et al.*[12], who reported the systemic resistance induced by cyclodipeptides, which sheds light on the potential of cyclodipeptides for the control of plant diseases.

Protein elicitors acts as signaling molecules during the pathogen entry and also they are the necessary components for the plant growth. Crude protein extracts were tested for growth parameter studies in muskmelon under *in vitro* and green house conditions. Among all the concentrations tested CP at 100 µg mL^{-1} concentration recorded highest seed germination of 90% and 2329.11 seedling vigor, at 6 h treatment *in vitro* and 42.35 cm plant height, 5 number of leaves, shoot fresh weight of 2.3 g, shoot dry weight of 1.30 g, root fresh weight of 0.43 g, root dry weight of 0.05 g and chlorophyll content of 4.16 mg g^{-1} at 6 h under greenhouse conditions. These results were better than the seeds treated with sterile distilled water. Similar studies were conducted by the similar result was reported by Nandini *et al.*[32] in pearl millet and Anupama *et al.*[34] in tomato, Abhayashree *et al.*[36] in

chilli with crude oligosaccharide elicitors. From these results it will be clear that not all the fungal borne chemical compounds will interfere with the plant growth, but they also enhance growth parameters in plants.

Plants have developed various weapons to fight against the un welcomed guests that affect their natural metabolic activities and growth. These weapons are in the form of mechanical barriers or chemical compounds that trigger the defense responses immediately. Proteins are one such elicitors act as defense reaction inducers in plants against entry of phytopathogens. Studies conducted by Wu *et al*.[12] revealed that cyclo (L-Pro-L-Pro) and cyclo (D-Pro-D-Pro) (where Pro is proline) could induce defense responses and systemic resistance in *Nicotiana benthamiana*. Treatment with the two cyclodipeptides led to a reduction in disease severity by *Phytophthora nicotianae* and Tobacco mosaic virus (TMV) infections compared with controls. Similar research by Wang *et al*.[39], explored the effects of protein and polysaccharide in *Meyerozyma guilliermondii* on active compounds in *Glycyrrhiza uralensis* Fisch adventitious roots. In their study, a responsive protein LSP1 was purified from the *Meyerozyma guilliermondii* since the excellent induction. The responsive protein LSP1 significantly activated the defense signaling, mitogen-activated protein kinases and extremely up-regulated the expression of defense-related genes and functional genes involved in glycyrrhizic acid biosynthesis. In the present study protein elicitors treated to the susceptible seeds of muskmelon to induce resistance against gummy stem blight disease. Along with the sterile distilled water treated negative control, fungicide (Mancozeb) treated seeds are also used as positive control to compare results of present study with crude protein elicitors.

CONCLUSION

From the results of this study, it can be concluded that crude proteins from *P. verruculosum* can be effective elicitor in inducing resistance against gummy stem blight disease. The results of the study showed that fungicide treatment offered a maximum of 94.03% disease protection, whereas CP at 100 μg mL^{-1} concentration recorded 74.49% disease protection.

SIGNIFICANCE STATEMENT

The study was aimed to find an ecofriendly alternative control strategy to the present chemical control method for GSB disease. The study exploits the disease controlling potential of the crude protein extracts from muskmelon rhizospheric fungus *P. verruculosum.* This elicitor can be further purified to identify the pure compound with antagonistic activity to enhance its disease protection over the systemic fungicides. This study not only control the major disease of muskmelon but also provides a better eco-friendly alternative to effectively control crop loss in muskmelon caused by GSB disease thereby reduced the environmental pollution.

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