ABSTRACT

Trichosanthes dioica Roxb. (Pointed gourd) is a dioecious, cultivated vegetable crop belonging to the family Cucurbitaceae. It is found to be cultivated throughout the plains of North and North-East India. It is also commercially cultivated in other Asian countries like, Pakistan, Sri Lanka and Bangladesh. In India, all parts of this plant had been traditionally used as food and in ayurveda to treat various disorders. It is one of the important cultivated vegetable crops in the plains of sub-Himalayan West Bengal. However, due to humid agro-climatic conditions, fungal infections are common in this region which leads to substantial economic losses. The farmers of the region are therefore largely dependent on the synthetic chemicals for disease management. Use of these synthetic chemicals not only derive a resistance in the pathogen but also disturb the normal soil-micro flora as well as create health hazards to human which necessitates research in alternative methods for limiting the fungal infections.

The understanding of molecular events of the host pathogen interaction leading to induction of resistance in a susceptible variety is of great importance in agriculture. In the present study, isolation of pointed gourd pathogens and their identification has been given first priority. Next, the genetic determinants of disease resistance were investigated. Finally, induction of disease resistance by some known elicitors was studied.

The objectives of the present study were as follows: 1) Isolation of major pathogens responsible for foliar and fruit diseases in *Trichosanthes dioica* available in sub-Himalayan West Bengal. 2) Pathogenicity tests of the isolated pathogens and assessment of disease incidence. 3) Physiological, morphological and molecular characterization of some pathogens. 4) Studies on disease resistance of *Trichosanthes dioica*. 5) Induction of plant defense with some signalling molecules against major isolated pathogens.

At the onset, pointed gourd fields of six districts of sub-Himalayan West Bengal *viz.* Darjeeling, Kalimpong, Jalpaiguri, Coochbehar, Alipurduar

and Uttar Dinajpur were surveyed from time to time for occurrence of diseases. Different types of symptoms were found on leaves, stems and fruits. The diseases were leaf blight, leaf spots (white as well as brown), fruit rot, fruit antracnose and whole plant necrosis. Altogether thirteen fungal pathogens could be isolated from different affected fields. Pathogenicity of the isolates was ascertained by pathogenicity tests through verification of Koch's postulates. Studies on morphological characters of the thirteen pathogenic fungal isolates revealed that four of them belong to the genus *Curvularia*, three of them were *Alternaria* sp., another three were *Fusarium* sp., one was *Aschochyta* sp., another one was *Periconia* sp. and the last one was *Colletotrichum* sp.

Seven different media *viz.* PDA, OMA, YEMA, MEA, PGDA, CDA, and RA were used to study the growth of the isolated fungi. From the results it was clear that PDA showed maximum growth of mycelia for all the tested fungi except *Colletotrichum* sp. which showed best vegetative growth in OMA medium. In all the cases, OMA was recorded as excellent medium for sporulation. Studies on the mycelial growth at different pH in PDA medium showed that mycelial growth occurred maximum at pH 5.5. A medium to low range of growth was recorded at pH 3.5 and pH 7.7. Further, the optimum temperature for the growth of all the isolated pathogens was 28°C.

For further confirmation of the identity of the isolated pathogens, molecular characterization was done where ITS of rDNA regions, partial 28S rDNA LSU regions and Actin genes of the isolated fungi were amplified through PCR, and obtained sequences were subjected to phylogenetic analysis following BLAST searches. GenBank accession numbers of 'ITS', '28S LSU' and 'ACT' genes have been given respectively in parentheses for each of the thirteen identified isolates: *Curvularia spicifera* (KX910098, KY411823, MN938365), *Fusarium equiseti* (KY411826, KY411824, MN938374), *Ascochyta medicaginicola* (MF447846, MF447845, MN938368), *Periconia macrospinosa* (MF447844, MF447843, MN938369), *Fusarium oxysporum* (MH842200, MH842203, MN938373), *Colletotrichum orbiculare* (MN006616, MZ314443, MN168524), *Curvularia lunata* (MN006628, MN006674, MN938366), *Curvularia aeria* (MN006621, MN006625, MN938367), *Curvularia verruciformis* (MN006618, MN006619, MN967012), *Alternaria alternata* (MN020527, MN010519, MN938370), *Alternaria destruens* (MN006678, MZ314458, MN938371), *Alternaria tenuissima* (MN006675, MN010576, MN938372) and *Fusarium equiseti* (MH744998, MH744997, MN938375).

Among the isolated pathogens, *C. spicifera*, *F. oxysporum*, *C. orbiculare*, *C. lunata*, *C. aeria*, *A. tenuissima*, *P. macrospinosa*, *C. verruciformis*, *A. medicaginicola* and *A. destruens* have been reported for the first time as a pathogen of pointed gourd. Morphological identification was found to be similar to that of molecular identification.

Till date no study has been conducted focusing on identification and characterization of Resistance (R) and Defense Related (DR) genes in the *T. dioica*. Further the evolutionary relationship of different categories of *T. dioica* R and DR genes within the family and across different plant species level is also lacking. However such studies are plenty in other cucurbitaceous plants. To fulfill the study, one R-gene *viz.* Pointed Gourd Resistance Gene Analogs (PG-RGA) and five DR-genes (PAL, Peroxidase, Glucanase, Chitinase, and PPO) were amplified from pointed gourd plant by using gene specific primers. After cloning, sequencing and BLASTn analysis, the genes were submitted to the GenBank. Phylogenetic analysis of R-gene was performed to identify its homologues within other genomes of cucurbitaceae. Results showed that in cucurbitaceous plants, both 'CC-NBS' and 'TIR-NBS' kind of motifs are present. The PG-RGA of *T. dioica* belongs to the group 'CC-NBS' and it showed a close sequence similarity with *Cucurbita moschata* sequences. The phylogenetic analysis of DR genes (PAL, Peroxidase, Glucanase, Chitinase, and PPO) was also done in comparison to their corresponding genes present on the genome of same and different families. Upon analysis it was observed that, the *T. dioica* DR gene sequence shared a common cluster with its identical gene within the family and showed a distant relationship with other species. This is a preliminary work regarding the R and DR genes present in *T. dioica,* their characterization and evolutionary study.

'Induced resistance' is an alternative approach for plant protection because it minimizes the use of toxic chemicals for disease control. Induced resistance is not based on direct defense activation by the inducing agent, but on a faster and stronger activation of inducible defense mechanism once the plant is exposed to the pathogen. This enhanced capacity to express basal defense mechanisms is called potentiation, sensitization or priming. There is a lack of information about defense induction in pointed gourd plant by these chemical inducers. In this study, seven different abiotic inducers *viz.* α-Aminobutyric acid (AABA), β-aminobutyric acid (BABA), γ-amino butyric acid (GABA), 2,1,3-Benzothiadiazole (BTH), Salicylic acid (SA), Abscisic acid (ABA) and Hydrogen peroxide (H_2O_2) were used to activate defense signaling in pointed gourd and induce defense related enzymes to elevate host resistance. The isolated strain of *F. equiseti* was used as pathogen. The entire experiment was performed with appropriate controls in four sets (untreated-uninoculated, untreated-inoculated, treated-uninoculated and treated-inoculated) for each inducer. Disease index values and levels of five defense related enzymes (PAL, Peroxidase, Glucanase, Chitinase and PPO) were estimated to correlate the effect of these inducers on host defense. Disease index data showed that BTH and ABA were better inducers to resist disease against the fungus *F. equiseti*.

In the present study, PAL activity was induced to maximum levels (2.5 fold increase) by treatment with SA. Significant increase of the enzyme was also observed in ABA and BTH treated plants. When level of Peroxidase was studied following application of seven inducers, about 5 fold increases in enzyme level was found to occur by ABA, BTH, AABA, GABA, BABA and H_2O_2 but SA did not induce the enzyme level significantly. Highest peroxidase activity was recorded in BTH and ABA-treated plants. β-1,3-glucanase was found to increase up to 1.5 fold after 7 days of inoculation. ABA and BTH treated plants also showed increased level of β-1,3-glucanase enzyme activity. In addition, Chitinase activity was found to be induced to highest levels by treatment of BTH and BABA. PPO activity increased almost 2.5 fold in case of BTH treated plants followed by ABA treated plants.

In order to confirm the over-expression of R and DR genes, semi-quantitative analysis by using Reverse Transcriptase PCR (RT-PCR) were done with the help of specific primers. To normalize the analysis, one housekeeping gene (26S rDNA) was taken into consideration. For this study, two experimental set-ups were placed, one with 'untreated-control' and another with 'inducer treated' plants. Three days post-treatment with the seven inducers, total RNA was extracted from all plants and measured spectrophotometrically. The 'inducer treated' plants showed more RNA concentration in comparison with 'untreated control'. The RT-PCR products and extracted RNA were subjected to agarose gel electrophoresis. Densitometric analysis of the developed gel was done by a computer software ImageJ version 1.5.2v. From the results it was evident that in 'inducer-treated' plants, R and DR genes showed more intensity in comparison to that of 'untreated control' plants.

The present study reports isolation of ten new pathogenic fungi from pointed gourd plants. Resistance Gene Analogs (RGA) of 'CC-NBS' type is also a new report in pointed gourd plants. Phylogenetic analysis of five different DR genes present in pointed gourd plants has also been done for the first time in the present study. In addition, use of chemical inducers to manage the fungal disease caused by *F. equiseti* could be established as an alternative to replace the harmful fungicides and to provide an efficacious control of the fungal disease.

PREFACE

The history of agriculture began thousands of years ago with the civilization of the Indus Valley. In 2016, agriculture and allied sectors like horticulture, forestry, animal husbandry, and fisheries together accounted for 15.4% of the country's gross domestic product (GDP). In 2018, 50% of the Indian workforce was employed in agriculture which contributed 17–18% to the GDP. India ranks first in highest net cropped area followed by the United States and China.

Large quantity of food crops and vegetables are destroyed annually due to fungal pathogens. Impact of such severe economical losses is also related to global poverty. According to statistics, the world harvest figures suggest that fungal diseases cause huge yield losses in the five most important crops; wheat, rice, maize, soybean and potatoes. If such losses were diminished, those crops would have been enough to feed at least 8.5% of the seven billion populations. Moreover, in a hypothetical incidence where these five crops were affected concomitantly, around 61% of the world's population would suffer a food shortage. Therefore, proper attention to control fungal diseases in different countries, specifically in developing countries needs to be given priority.

As fungal diseases are a major threat to crop production, the application of fungicides to control fungal diseases is often considered necessary to secure the worldwide food supply. Furthermore, deliberate use of fungicides change the soil conditions and give rise to invasive fungal species. Most importantly it helps in development of fungicide resistance. The excessive use of fungicides causes health hazards to all the living creatures inhabiting both land and water, as it can enter aquatic ecosystems via drift, drainage and surface runoff from agricultural use. Despite the various risks in living systems and the environment due to excessive use of fungicides, the effects of fungicides have received far less attention. Under these circumstances, there is a need for constant search for new environmental friendly fungicides, effective measures to prevent fungicide resistance, and more importantly novel treatment strategies by utilizing plant's own defense mechanisms through understanding plant-pathogen interactions.

Pointed gourd (*Trichosanthes dioica*) is one of the most consumed vegetable in the Asian tropical countries. It is mainly cultivated in India, Bangladesh, Pakistan, Myanmar, Nepal and Sri Lanka. In India, a total of 2,52,000 metric tons of pointed gourd was harvested from 18,000 hectares of land during 2016-2017. Different parts of the plant are used in a number of Ayurvedic preparations by the folk practitioners. Several fungal diseases have been reported to cause considerable damage to pointed gourd production in India. These include downy mildew caused by *Pseudoperonospora cubensis,* fruit rot by *Pythium aphanidermatum* and *P. cucurbitacearum*, sclerotinia stem rot by *Sclerotinia sclerotiorum,* fruit and vine rot by *Phytophthora melonis,* anthracnose by *Colletotrichum capsici* etc.

Increasing fungal attacks necessitated the use of different types of fungicides. The compulsion of alternative environment-friendly control methods are being understood by the researchers and policy makers. For this, proper understanding of the complex defense mechanisms of plants, their interaction with applied defense inducing molecules and the invading fungal pathogens are necessary in order to increase the efficiency and realize the true potential of sustainable disease control methods.

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