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GEOGRAPHICAL DISTRIBUTION OF MYROTHECIUM LEAF SPOT DISEASE OF *MOMORDICA CHARANTIA* L. CAUSED BY *MYROTHECIUM RORIDUM* TODE IN AGRO-ECOLOGICAL ZONES OF PUNJAB, PAKISTAN

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Abstract

A series of field surveys were carried out during July-October 2012-14 for the development of disease distribution map and updating the index of *Myrothecium* leaf spot of *Momordica charantia* (Bitter gourd) in Punjab province. A total of 29 districts were surveyed belonging to 9 sub agro ecological zones of Punjab. The index was calculated on the basis of incidence, prevalence and severity of the disease. The information from stakeholders was gathered through field scouting, formal and informal discussions. Data on socioeconomics was collected by a structured questionnaire. The symptomatic plants and soil specimens were collected form diseased field and transferred to lab for onward studies on host-pathogen characterization and management. Infection development on the plant was investigated on a (0-5) visual severity rating scale. Fungus was isolated, identified on morphological and molecular characteristics as *Myrothecium roridum* and cultures were deposited to First Fungal Culture Bank of Pakistan (Accession # FCBP 1155) and Leibniz-institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany (Accession # DSM 28971). Data gathered highlighted dominance of the disease in mixed cropping zone of Punjab province. Highest disease index (31%) was recorded in mixed cropping zone and lowest (3%) in D.G khan zone. However its severity may vary due to adopted cultural and chemical practices by individual farmers. The investigations strengthen the involvement of irrigation technique, soil type and cropping history in introduction and sporadic occurrence of the disease.

Key words: *Myrothecium roridum*, Incidence, Prevalence, Severity, *Momordica charantia*.

Introduction

Bitter gourd (*Momordica charantia*) is an important cucurbitaceous vegetable in Asia, Africa and South America. Bitter gourd has high nutritive and medicinal value and an excellent source of iron, vitamin C potassium, calcium, magnesium, protein, and dietary fiber (Islam *et al*., 2011**)**. Medicinal value of plants is well documented in fighting against diseases and disorders (Zahra *et al*., 2016). Phenolic compounds, antioxidants and antimutagen properties of bitter gourd contribute in its medicinal importance. In Pakistan it is cultivated twice a year as spring and summer crop during Jan-March and June- July with an annual production of 56994 tons (Anon., 2009-10). Average yield in Pakistan is 8-10 tons/ ha which is much lower than neighboring countries like China and India where it is 20-30 tons/ha (Anon., 2011). Besides policy and management constraints, biotic and abiotic stresses are responsible for lower yield. Bitter gourd is susceptible to a wide range of fungal, bacterial and viral diseases. Among biotic stresses, fungal diseases play a major role in deterioration of yield and produce quality.

Myrothecium leaf spot disease has been recognized as a seriously damaging fungal disease of bitter gourd plant in Punjab. It was first reported to infect bitter gourd crop during 1988 in Pakistan (Ali *et al*., 1988). The disease is characterized by appearance of water soaked minute spots that are dark brown to black in color (Kim *et al*., 2003). These spots vary in shapes from round to irregular and may present anywhere on leaves. These spots coalesce on later stages to form blighted areas on the leaves (Belisario *et al*., 1999). Irregularly shaped black sporodochia can form with a white fringe of mycelium. These spore structures appear in concentric rings within the necrotic areas and seen on the leaf undersides. *Myrothecium* leaf spot most frequently appears on wounded areas of leaves such as tips and breaks in the main vein which occurs during handling. Sultana & Ghaffar, (2009) examined pathogenesis of *M. roridum* of bitter gourd.

There is scarcity of information on distribution, ecology and other relevant aspect of Myrothecium leaf spot disease (MLSD) in Pakistan. Present studies are designed to evaluate the disease index of Myrothecium leaf spot disease of bitter gourd in various agroecological zones of Punjab, Pakistan. The main objective of the surveys was to assess status of pathological constraints and associated factors under the light of baseline survey.

Materials and Methods

Survey: The surveys were conducted on a scheduled plan from April 2012 to September 2014 at seedling, vegetative, flowering and fruiting growth stages of the crop. It was conducted on early spring (February-March) and late summer (mid June – mid July) seasoned crops. Information on cropping history, input source, and crop production and protection technology was gathered on a structured questionnaire. Structured questionnaire was distributed among the stakeholders. Information collected from the surveys was analyzed and expressed in percentage to evaluate the consumption, quality census of the consumers and market status. Preliminary survey was conducted during July-September 2010. The key objective of this survey was to construct questionnaire,

survey scheduling, route, basic information on crop and disease with respect to its stake holders as farmer, representatives' public and private sector organization engaged in marketing and R&D sector for vegetable.

In Pakistan vegetables are grown on small pieces of land and sometimes small fields are not in proper sequence or rectangular shape so 0.25 hectare was considered as basic sample unit. A total of 319 locations from 11 sub agro-ecological zones covering 24 districts and 117 fields comprising on 0.25 hectares from farmers' fields, demonstration plots of Agriculture Extension Wing of Punjab Agriculture Department were visited. To achieve real field representation, fields were examined in cross, zigzag or diagonal fashion depending upon geometry of the field.

Disease assessment: Disease assessment was made on disease prevalence, incidence, severity and percent disease index. Formulae used for the calculations are given below. Prevalence percentage of the disease was calculated on the basis of the number of locations showing disease in an area; whereas, Percent disease incidence was noted on leaves/plant infected from five spots in a field. Data for severity of disease was recorded on a 0-5 visual severity rating scale (VSRS, Fig. 1). Disease index of an area was calculated on sum of all numerical categories of disease severity divided by number of samples.

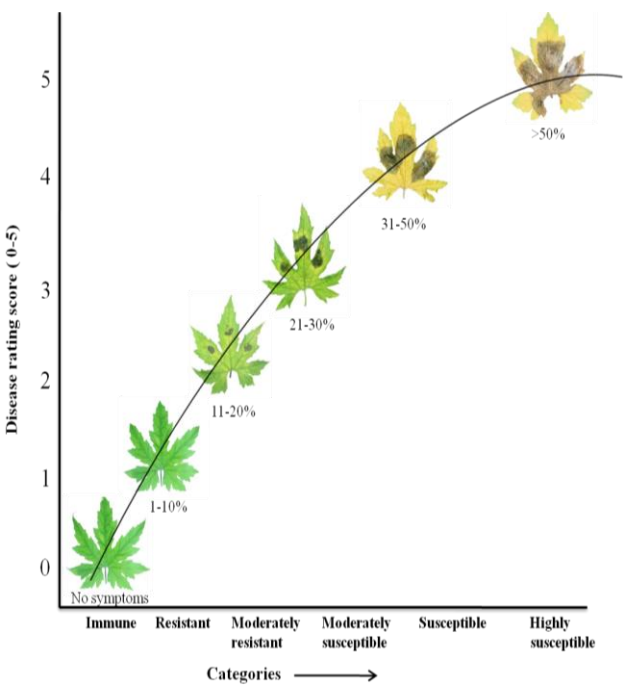

Fig. 1. Illustration of 0-5 visual severity rating scale (VSRS) of MLS disease of bitter gourd.

$$\text{Percent prevalence (PP)} = \frac{\text{Locations with MLS disease}}{\text{Total locations surveyed in that particular area}} \times 100$$

$$\text{Percent disease incidence (PDI)} = \frac{\text{Number of sample with MLS symptoms}}{\text{Total number of samples}} \times 100$$

$$\text{Percent disease index (PDIn)} = \frac{\text{Sum of all numerical categories}}{\text{Total number of samples}} \times \frac{100}{5}$$

Specimen collection: To find out the association of *Myrothecium roridum* with Myrothecium leaf spot disease of bitter gourd, leaves showing the characteristic Myrothecium leaf spot disease symptoms were collected, sorted individually, packed in cellulose bags and labeled with necessary information regarding location, time, date and grower identification. Collected specimens were transported to Seed and Postharvest Pathology Laboratory, Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore where isolation, identification and lab scale multiplication of the single spore cultures of the associated fungi were carried out.

Isolation of associated fungi: Collected samples were examined under stereoscope and infected leaf, stem and fruits with typical MLS symptoms were processed further. Potato dextrose agar medium (PDA) was used for isolation in 5 replicates for each test sample. Samples were washed under running tap water and placed overnight at 4°C, cut into 1 cm^2 pieces, surface sterilized with 1% sodium hypochlorite solution for five minutes followed by thorough washing with autoclaved double distilled water and aseptically transferred on 90mm Petri plates containing PDA medium. These Petri plates were incubated at 25°C for 3-5 days. The isolated fungus was purified by transferring actively growing mycelium from the colony margins.

Identification of fungus: Single spore cultures were produced on fresh PDA and examined for its morphology and taxonomy. Detailed taxonomic studies were carried out under compound microscope at 100X and 400X. Key identification features of *Myrothecium roridum* Tode i.e., colony color and shape, mycelia morphology, sporodochia production, conidia formation, conidia size and shape were studied as described by Mycobank, Korea (Mycobank # 142164). The confirmed colonies of *M. roridum* were transferred on 90mm PDA Petri plates, incubated on PDA at 25 °C for culture submission to First Fungal Culture Bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Lahore.

Application of Koch's postulates: Pathogenicity was confirmed by application of Koch's postulates before initiating towards the series of experiments. Twelve centimeter disposable glasses were filled with sandy loam soil and sterilized by 40% commercial formaldehyde. Isolated fungus was evaluated for its pathogenicity on three weeks old plants of *Momordica charantia*. Leaves were sprayed with spore suspension till run off and examined for symptoms development after every 24 hrs for 7 days. The experiments were carried out in growth room at temperatures of 25-30°C.

Culture authentication: Genomic DNA was extracted from fungal mycelia using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA amplification was performed using Polymerase Chain Reaction (PCR) in three biological replicates. Universal primer pairs, ITS4 (TTCCTCCGC TTATTGATATGC) and NS1 (AACTTAAAGGAA TTGACGGAAG) following standard PCR procedures with minor modifications. Sequence analysis was done by the LGC AGOWA Ltd. at LGC Genomics GmbH, Berlin. Sequences obtained were compared with all sequences of ITS region in the GenBank closest sequence similarity by using BLAST.

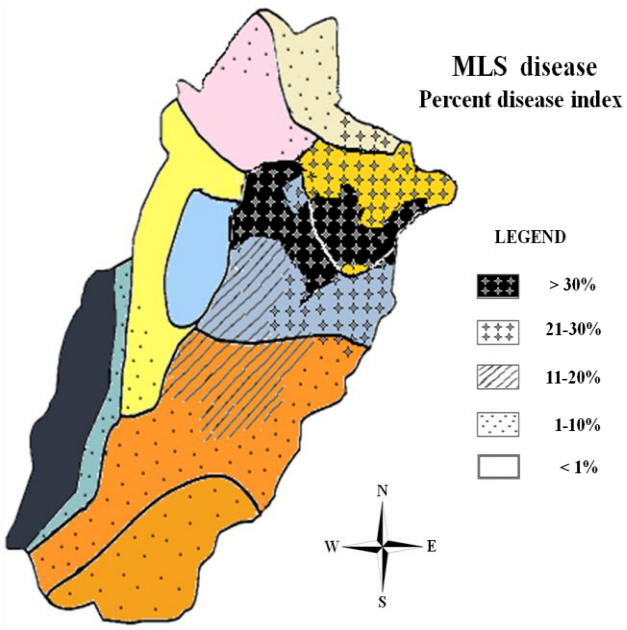

Fig. 2. Geographical distribution map of Myrothecium leaf spot of bitter gourd in agro-ecological zones of Punjab, Pakistan during 2012-14. (Modified PARC, http://old.parc.gov.pk/Maps/AgroEcoPunjab.html)

Results

Geographical distribution of MLS in Punjab: Disease was widely distributed and none of the area or cultivar was found disease free in canal irrigated plains and Barani areas (Fig. 2). The disease was observed at every growth stage of the plant and cumulative description of the surveys conducted during 2012, 2013 & 2014 reveals the highest prevalence, disease incidence and severity and disease index for Myrothecium leaf spot (MLS) disease in mixed cropping zone of Punjab (Table 1).Mean prevalence of MLS disease was consecutively recorded 100% for the surveyed years in mixed cropping zone whereas in rice zone, 36, 32 and 42% for the year 2012, 2013 and 2014 respectively were recorded. Barani zone showed prevalence range of 14 to 17% and the highest 17% was recorded during 2014 whereas cotton zone exhibited range of 14 to 22% and the highest 22% was recorded in during 2013. The least prevalence recorded in DG Khan Zone was 11, 19 and 14% during the years 2012, 2013 and 2014 respectively. Disease index ranged 3-31% during 2012-2014 with the highest index of 31 % in mixed cropping zone and the least of 3 % in DG Khan zone during 2014. Disease incidence ranged between 8-13% with severity range of 0-2 on 0-5 visual severity rating scale. MLS disease was not observed in Thal irrigated region comprising of Mianwali, Bhakkar and Khushab districts and Marginal land comprising on Bahawalpur district throughout the survey period.

Detailed monitoring of specific sites was conducted in five major bitter gourd producing districts of central Punjab viz., i.e., Lahore, Kasur, Faisalabad, Sargodha and Jhang, considered as major bitter gourd production areas of mixed cropping zone. Disease incidence ranged between 42-50% with severity range of 1-4and 28 % disease index for mixed cropping zone (Table 2). Among the districts within mixed cropping zone, Lahore showed the highest disease incidence i.e., 59% and disease severity range of 1-4 on scale. Sargodha, Kasur and Faisalabad showed a moderate response with 48, 45 and 33 % disease incidence and 1-4, 1-3 and 1-2 severity scale respectively. The least disease incidence of 29% with 0-2 disease severity and 10% disease index was recorded at District Jhang in mixed cropping zone (Table 2).

Table 1. Geographical distribution of *Myrothecium roridum* in agro ecological zones of the Punjab, Pakistan.

Zones	Year	PP	PDI	DSR (0-5)	PDIn
DG Khan	2012	11.35	8.05	0-2	3.27
	2013	19.42	13.30	0-2	4.13
	2014	14.26	11.23	0-1	3.23
Barani (Rain fed)	2012	16.15	12.28	1-3	4.28
	2013	14.11	9.21	1-2	6.1
	2014	17.41	13.24	0-2	5.83
Rice zone	2012	36.43	33.16	1-3	14.78
	2013	32.14	26.08	1-2	15.12
	2014	42.07	35.22	1-3	16.47
Mixed zone	2012	100	45.20	2-4	28.97
	2013	100	42.31	1-4	25.43
	2014	100	49.22	1-4	30.72
Cotton zone	2012	14.31	14.09	1-2	5.32
	2013	21.19	18.19	0-2	5.66
	2014	19.23	15.19	1-2	4.07
Marginal land	2012	0	0	0	0
	2013	0	0	0	0
	2014	0	0	0	0
Thal region	2012	0	0	0	0
	2013	0	0	0	0
	2014	0	0	0	0

PP= Percent prevalence; PDI= Percent disease incidence; DSR= Disease severity range; PDIn= Percent disease index

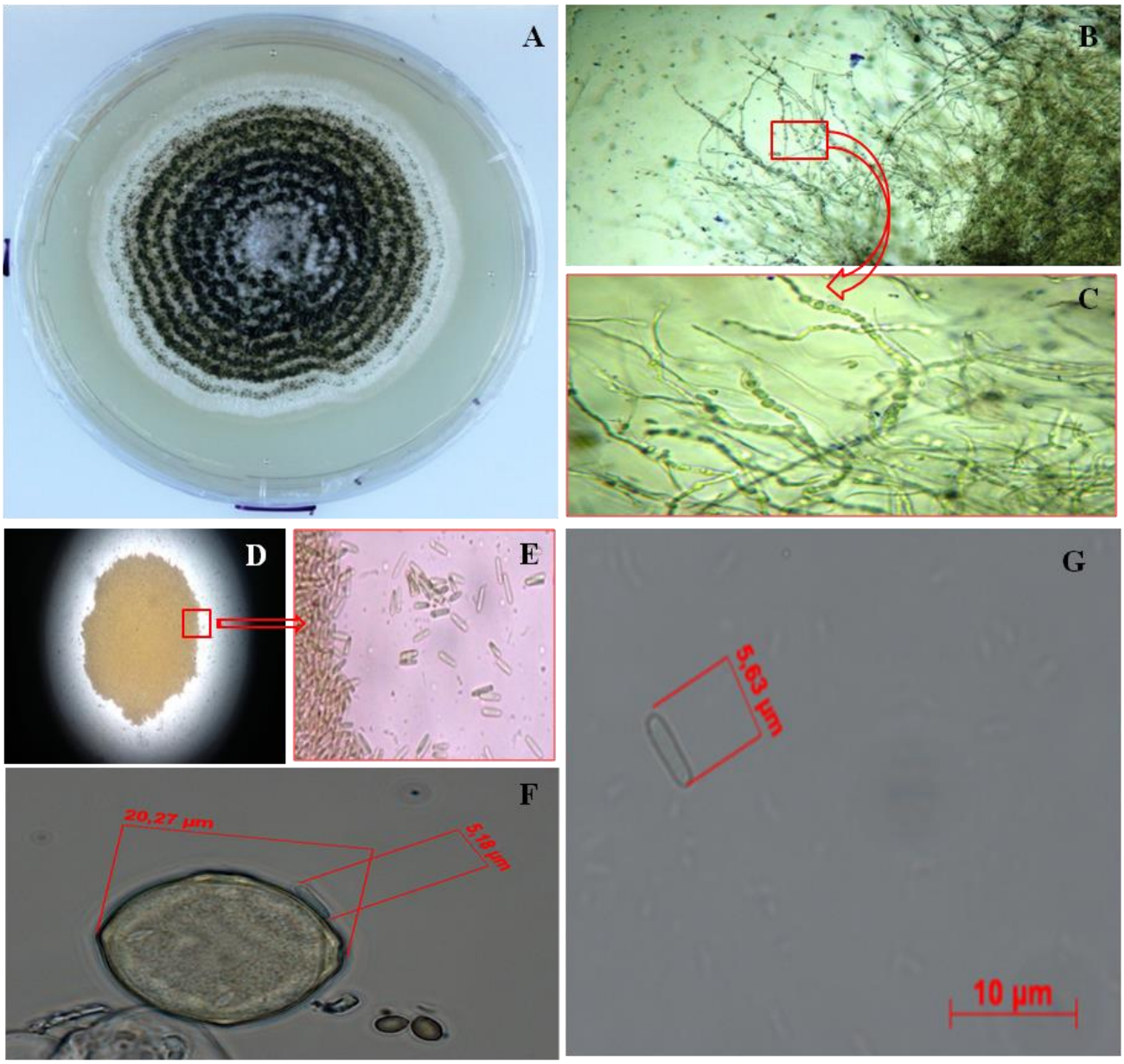

Plate 1. Characteristics identification features of *Myrothecium roridum*. A: colony on PDA medium; B: mycelia at 100X; C: conidia formation; D & E: sporodochia at 40X and spores; F and G: single spore of *M. roridum* at 1000X (Axioplan 2, Germany).

Table 2. Geographical distribution of *Myrothecium roridum* during 2012-14 in districts of mixed cropping zone of the Punjab, Pakistan.

District	PDI ± SE	DSR (0-5)	PDIn ± SE
Lahore	59.07 ± 0.23	1-4	31.43 ± 0.19
Kasur	44.56 ± 0.11	1-3	27.04 ± 0.07
Faisalabad	33.17 ± 0.28	1-3	23.14 ± 0.20
Sargodha	48.22 ± 0.29	1-4	31.05 ± 0.25
Jhang	28.73 ± 0.13	0-3	10.27 ± 0.17

PDI= Percent disease incidence; DSR= Disease severity range; PDIn= Percent disease index; SE= standard error

Isolation and identification studies of *M. roridum*: A total of 317 diseased bitter gourd specimens showing characteristics symptoms of infection on stems, leaves and fruit as well as rhizospheric soil of infected plants were collected from different location of the Punjab province. Diseased samples were inoculated on PDA medium for isolation of associated fungi Single spore cultures of the isolated fungi were obtained and studied for their characteristics (Plate 1). Pathogenicity of the pathogen *M. roridum* was confirmed by adopting Koch's postulates following the leaf inoculation technique.

DNA was extracted and PCR product was prepared according to the manufacturer direction (Qiagen, Hilden, Germany). The sequence of 1200 bp fragments of ITS rDNA PCR product was determined by LGC AGOWA. The sequence was used to search Genbank database of NCBI and we found that it has 99% similarity with *M. roridum* gb: strains BBA 71015 (AJ3020010) and BBA 67679 (AJ301995). *M. roridum*, 3-5 days old cultures were submitted to First Fungal Culture Bank of Pakistan (FCBP accession no. 1155) and Leibniz-institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Accession # DSM 28971).

Discussion

Earlier consideration for *Myrothecium roridum* in Punjab, Pakistan was a weak parasite of bitter gourd (Ali *et al*., 1988). This hypothesis was attributing to monoculture cropping and poor management strategies especially the unjustified use of agronomic inputs has made sporadic occurrence Myrothecium leaf spot on bitter gourd. In this regards very little information is available on disease epidemiology and distribution pattern. Survey is considered as an important step for initiation of a project, it provides reliable information on statistical updates and involvement of socio-economic factors associated with the issue. While designing initial project draft, reliability of survey findings depends upon survey methodology, sample size, fluctuation intensity and measuring scale. Key objectives for present field investigations were to update disease statistics, analyzing role of production technology and social factors in disease spread. During field scouting disease specimens were also collected to get single spore culture population of the isolates for onward investigations mentioned for disease diagnostics and management. In the present investigation, guidelines of Hoagland *et al*. (2007) and Michereff *et al*. (2011; 2012) were observed. In addition to above guide lines reliability of socioeconomic factors was cross examined by distribution of a structural questionnaire, formal and informal interviews with farmers, public and private sector stake holders engaged with crop production and marketing. Major attention was focused on spring crop which is sown during mid February to mid April and harvested May-July.

Vegetable are cultivated as regular feature on 10 days interval on small holdings. This interval is in continuous cultivation on smaller units to keep in touch with the market for sustainable farm income. Therefore at the same time different growth stages of the crop can be seen in the adjacent fields. Before initiation of the survey Punjab province was divided in four agro-ecological zones viz., irrigated planes, Barani (Rain fed) region, Thal region and Marginal Land. While conducting the survey, attempts were made to ensure maximum representation of a soil type and production technology observed in a region. The cumulative assessment of surveys conducted during 2012, 2013 and 2014 exhibited highest disease index of 29, 25 and 30% respectively in the mixed cropping zone. Whereas the least disease index of 3.27, 4.13 and 3.23% during 2012, 2013 and 2014 respectively was recorded in DG Khan zone. Disease severity was ranged 1-4 on visual severity rating scale (VSRS) for mixed cropping zone while in DG Khan irrigated; it ranged from 0-2 during the surveyed years. The mixed cropping zone consists of Lahore, Faisalabad, Kasur, Gujranwala and Jhang districts. Significant variation prevails for meteorological conditions, production technology, market approach investment trends and crop protection strategies. In mixed cropping zone soil is fertile and intensive cultivation culture prevails in general. Cultivation of vegetable in tunnels makes it more susceptible because fungal inoculums and availability of favorable environmental conditions make situation worst (Powell *et al*., 2013). Due to higher return, market hub farmers prefer to cultivate and invest on crop protection. On the other hands DG khan and Bhakar, areas at certain sites we could not observe the disease or it has low intensity. In these areas no strong background for cultivation of vegetables on commercial scale exists because of its dry hot climate with sandy loam soils. Whereas on the west bank of DG khan link canal, low rainfall and poor quality sandy loam soil generally prevails. Adoption of best approaches for on-farm irrigation management and innovative crop production technologies for such remote areas where lesser or absence of disease is recorded, could result in good quality production of vegetables with higher yield (Si *et al*., 2017).

Conclusion

Keeping in view disease statistics and analysis of field and market sociology it is suggested that these disease free or lower index areas should be promoted along with judicious application of inputs for vegetable especially bitter gourd cultivation.

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