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Genetic variation among isolates of *Bipolaris maydis* using RAPD_PCR

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Abstract

Maydis Leaf Blight prevalent in many parts of India is a major threat to maize cultivation when grown in warm and humid climates. The pathogen responsible is *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker. Ten isolates of the fungus were selected from different geographical regions in India for the present study. Preliminary primer screening was carried out using 32 primers from OPM, OPB and OPN series (Operon Technologies, Inc., USA) for molecular variation analysis. Out of 32 primers used for amplification of DNA of all isolates of *B. maydis*, two resulted in either sub-optimal or non-distinct amplification products. The similarity coefficients subjected to UPGMA-SAHN to produce a dendrogram, resulted in two major clusters, which separated Bm-6 from rest of the isolates, which were grouped in two major cluster, one having Bm-3 and Bm-2 and the second one having the rest of the isolates in two sub clusters. The first sub cluster included Bm-4 and Bm-7 while Bm-1, Bm-5 and Bm-9 were grouped in second sub cluster. Isolates Bm-8 and Bm-10 were different from rest of the isolates in the second sub cluster thus remained one grouped. In present investigations though significant correlation between the genetic similarity and geographical origins could not be established.

Keywords: maydis leaf blight; India; molecular markers

Riassunto

Il seccume fogliare del mais è una malattia diffusa in molte aree dell'India con clima caldo-umido. Il patogeno responsabile è *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker. Dieci isolati del fungo sono stati selezionati per il presente studio da diverse aree geografiche dell'India. Uno screening preliminare è stato effettuato per l'analisi della variazione molecolare usando 32 primer delle serie OPM, OPB e OPN (Operon Technologies, Inc., USA). Dei 32 primer utilizzati per l'amplificazione del DNA da tutti gli isolati di *B. maydis*, due hanno dato prodotti di amplificazione sub-ottimali o non distinti. Il dendrogramma ottenuto dai coefficienti di similarità con il metodo UPGMA-SAHN è risultato in due cluster principali, uno con Bm-3 e Bm-2 ed un secondo con il resto degli isolati raggruppati in due sottogruppi. Il primo sottogruppo ha incluso Bm-4 e BM-7, mentre Bm-1, Bm-5 e BM-9 sono stati raggruppati nel secondo sottogruppo. Gli isolati Bm-8 e Bm-10 sono risultati diversi dal resto degli isolati, andando a costituire un gruppo a parte. Nel presente studio non è stato possibile stabilire una correlazione significativa tra la similarità genetica e le origini geografiche degli isolati.

Parole chiave: seccume fogliare del mais; India; marker molecolari

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Introduction

Maize has occupied an important place not only in India but elsewhere as well, due to its great significance as food, feed and industrial utilization. In India 61 diseases have been reported on maize (PAYAK and SHARMA, 1985). Among them Maydis Leaf Blight (MLB) prevalent in many parts of India, is a major threat to maize cultivation when grown in warm and humid climates. The disease has its presence in Jammu and Kashmir, Himachal Pradesh, Sikkim, West Bengal, Meghalaya, Punjab, Haryana, Rajastan, Delhi, Uttar Pradesh, Bihar, Gujarat, Maharashtra and Andhra Pradesh. Maydis Leaf Blight is incited by a fungal pathogen earlier referred to as *Drechslera maydis* (Y. Nisik. & C. Miyake) Subram. & B.L. Jain, but during the course of time the international scientific community has changed the generic name of the pathogen to *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker. Thus here after the name of the pathogen is cited as *Bipolaris maydis.*

In recent years, plant pathologists interested in genetic variation in pathogen populations have adopted the use of molecular markers as population genetics tools. Motivating this shift has been the availability of a myriad of molecular techniques which makes the quantification of genetic variation a relatively straightforward endeavor (MICHELMORE and HULBERT, 1987; BROWN, 1996). Molecular markers such as allozymes (LEUNG and WILLIAMS, 1986; GOODWIN et al., 1993), restriction fragment length polymorphisms (RFLP) (MCDONALD et al, 1990; KOHLI et al, 1992, MILGROOM et al, 1992) and random amplified polymorphic DNA (RAPD) (HAMELIN et al, 1994; PEEVER and MILGROOM, 1994) have been extensively used to characterize pathogen populations. More recently, amplified fragment length polymorphisms (AFLP) (MAJER et al, 1996; MUELLER et al, 1996) have proven to be highly polymorphic and robust markers and will likely be used extensively with plant pathogenic fungi in the future. In contrast to virulence and fungicide resistance markers, molecular markers are presumed to be selectively neutral and therefore may be used to study evolutionary processes in addition to selection (MILGROOM and FRY, 1997).

The RAPD method was shown to be reproducible and to yield more objective results than symptom observation. In addition to morphological characteristics it can be rapid and reliable tool to identify a fungal isolate. RAPD patterns were extremely useful to identify false classified isolate. Clearly, new approaches are needed to improve the taxonomy of *Bipolaris* and changes should reflect their genetic relations. Recently, the phylogenetic relationships reflected in similarities at the level of DNA sequences have been adopted for comparison among many phenotypically uncertain (BAUN, 1992).To evaluate such similarities, DNA base composition, DNA homologies have been studied and compared (MULLANEY and KLICH, 1990; METZENBERY, 1991). Keeping these concerns in view, the present study was initiated to study genetic variation in isolates of *B. maydis* by PCR-RAPD.

Materials and Methods

Ten isolates of *Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker were selected for the present study. These isolates were collected from different geographical regions in India. The isolates with their designation (Bm) are listed in Tab. 2 and Fig. 1.

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Tab.1 Place of collection and designation given to individual isolates of *Bipolaris maydis* (Bm)
Tab.1. Elenco, luoghi di provenienza e sigle degli isolati di *Bipolaris maydis* (Bm) utilizzati nel lavoro

Place of collection	Location (State)	Designation to isolates
Changla	Udaipur (Rajasthan)	Bm-1
Jashipur	Orissa (East) (Orissa)	Bm-2
Assam	Assam (East) (Assam)	Bm-3
Almora	Almora (Uttarakhand)	Bm-4
Nia-Village	Udaipur (Rajasthan)	Bm-5
IARI	Delhi (New Delhi)	Bm-6
Karnal	Karnal (Haryana)	Bm-7
Gorang	Udaipur (Rajasthan)	Bm-8
Pantnagar	Pantnagar (Uttarakhand)	Bm-9
Undithal	Udaipur (Rajasthan)	Bm-10

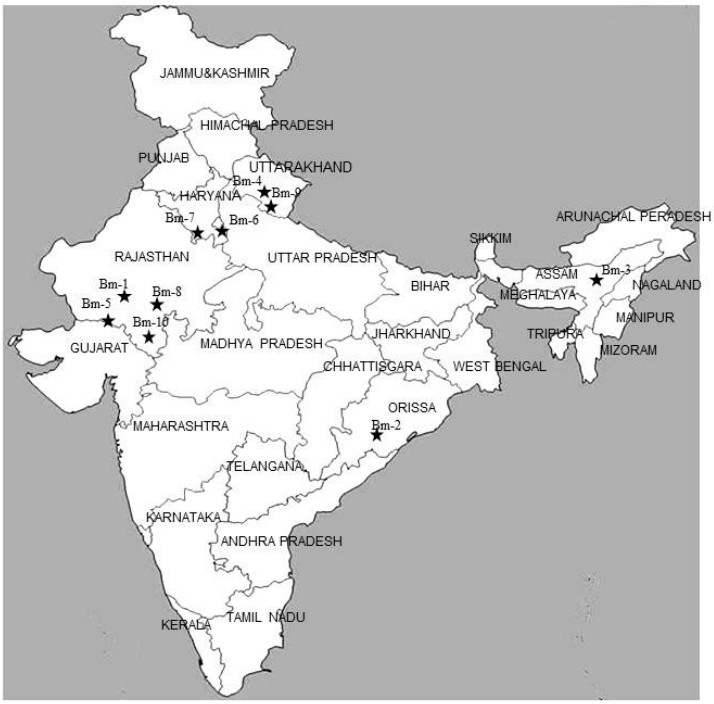

Fig. 1 Map of India with places where isolates were collected
Fig.1 Mappa dell'India con i siti di provenienza degli isolati

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Fungal multiplication

Potato dextrose broth was used to get mycelial growth of fungus for extraction of DNA. One hundred and fifty ml of medium was dispensed in 500 ml conical flasks and sterilized at 15 p.s.i. for 20 min. Each flask was inoculated with 6 mm diam. mycelial disc of the fungus, taken from the actively growing single spore cultures of different isolates growing on PDA. The inoculated flasks were incubated for 10 days at 28±1° C. At the end of incubation period, the mycelial mats were harvested by filtering through Whatman paper n.1 filter paper, washed with sterilized water thrice, blot dried and stored in aluminum foils at –20° C.

Genomic DNA isolation

DNA extraction was done by procedures given by MURRAY and THOMPSON (1980) and ZOLAN and PUKILLA (1985) with slight modifications. Mycelial mats were weighed (1 g), ground in liquid N_2 in pre-chilled pestle and mortar, taking care not to let the samples to thaw. Powdered mycelium was transferred into centrifuge tubes carrying 25 ml of pre-heated (65° C) 2 % CTAB extraction buffer to make a slurry. The tubes were incubated at 65° C for an hour and stirred occasionally with the help of sterile glass rod. Equal volume of chloroform isoamyl alcohol (24:1) was added to each tube and mixed gently. Samples were centrifuged at 10,000 rpm for 10 min. at room temperature. Upper aqueous phase was separated and precipitated with 0.6 vol. of ice-cold isopropanol and 0.1 vol. of 3M-sodium acetate (pH 5.2) and centrifuged at 15,000 rpm for 15 minutes. The pellets obtained were washed with 70% ethanol and kept for drying at room temperature (25° C). Total nucleic acid obtained was dissolved in sterile distilled water and stored at –20° C in small aliquots.

Purification and quantification

RNase treatment was given to remove RNA from the total nucleic acid. 2 µl of RNase from stock solution was added to nucleic acid extracted and incubated at 37° C for an hour. DNA concentration of samples and purity were determined by taking ultra-violet absorbance at 260 nm and 280 nm in a NanoDrop™ 8000 spectrophotometer and rechecked by running samples on 1% agarose along with 1kb molecular weight marker (MBI, Fermentas).

Random Amplification of Polymorphic DNA

Optimization of Polymerase Chain Reaction (PCR)

Composition of PCR reaction was optimized by varying the concentration of template DNA (25, 50, 75 and 100 ng), Taq DNA Polymerase (0.5, 1.0 and 1.5 units) and $MgCl_2$ concentration (3, 5, and 7.5 mM). The standardized amplification assay was as follows: Template DNA 25 ng; Taq DNA Polymerase (Banglor geni) 0.5 units; $MgCl_2$ 5 mM; dNTP (Genei) 100 µM each of dATP, dGTP, dCTP, dTTP, primer (Operon Technology) 1µM, Buffer (Genei)-1x in a reaction volume of 25 µl. Different PCR protocols given by PASCUAL et al., (2000) and LEE and TAYLOR (1990) were tested for obtaining best amplification of nucleic acid of the isolates under investigation. The Polymerase chain reaction was performed using Bio-Rad Gen cycler™, with the following temperature profile: the initial denaturation at 94° C for 2 min followed by 45 cycles of three steps of denaturation at 92° C for 1 min. annealing at 37° C for 1 min. and extension at 72° C for 2 min. followed by final one elongation cycle of 72° C for 5 min.

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Primer screening and selection

Preliminary primer screening was carried out using 32 primers from OPM, OPB and OPN series (Operon Technologies, Inc. USA) for molecular variation analysis. Out of 32 primers used for amplification of DNA of all isolates of *B. maydis*, 2 resulted in either sub-optimal or non-distinct amplification products. Therefore, these were discarded and remaining 30 were used for PCR amplification, which were found to give reproducible and scorable bands with high percentage of polymorphism. PCR amplification with each of the primers was done twice before scoring for presence and absence of bands. Each amplification product was considered as RAPD marker and recorded across all samples. The RAPD pattern of each isolate was evaluated assigning character state 1 to all the bands that could be reproducibly detected in the gel and 0 for the absence of band at same locus. Data was entered using a matrix in which all observed bands or characters were listed.

Analysis of genetic diversity based on RAPD markers

Estimation of genetic distance

The data matrix thus generated was used to calculate Jaccard's similarity coefficient for pair wise comparisons. For this purpose, NTSYS-pc Version 2.0 (ROHLF, 1998) software was used.

Cluster analysis

The RAPD data were analyzed using the Statistical Package for the Social Sciences (SPSS) software, 2nd edition (WILKINSON et al., 1992), through which similarity coefficients were calculated and a dendrogram for genetic distances constructed. In the present study, cluster analysis was done using commonly adopted clustering algorithm in genetic diversity analysis, namely UPGMA (Unweighted Pair Group Method using Arithmetic averages) (CRISCI and ARMENGOL, 1983). The resultant similarity matrices based on Jaccard's measure were further analysed by performing sequential, agglomerative, hierarchical and nested clustering (ROHLF, 1998) using the UPGMA method. The results of clustering were plotted in the form of dendrograms.

Results and Discussion

Primer survey was carried out using 32 primers from OPM, OPB & OPN series of Operon Technologies, USA. It is indicated that out of 32 primers screened for amplification of DNA of isolates of *B. maydis* 30 were found to give reproducible and scorable bands with high percentage of polymorphism (94 %). All the bands scored were polymophic in the amplification products maximum polymorphism was observed in OPN1, OPN3, OPN6, OPN8, OPN14, OPN15, OPN17, OPN 18 and OPN20 with 100 percent polymorphism as total number of bands obtained were polymorphic for these primers, while primers OPM19, OPM11, OPM2, OPM8, OPM12, OPM13 and also OPB6 gave a good indication of the variability, as the also resulted in more than percentage polymorphism (Fig. 2).

Scoring and analysis of RAPD-PCR amplification

The total number of bands obtained was 346 out of which 328 were polymorphic. Genetic variation observed in the study was maximum between isolates from Bm-6 and Bm-5 (66.2 per cent), closely followed by isolates Bm-6 and Bm-3 (64.4 per cent). Isolates from Bm-1 and Bm-5 were genetically closer than any other isolates with 60 percent, similarly.

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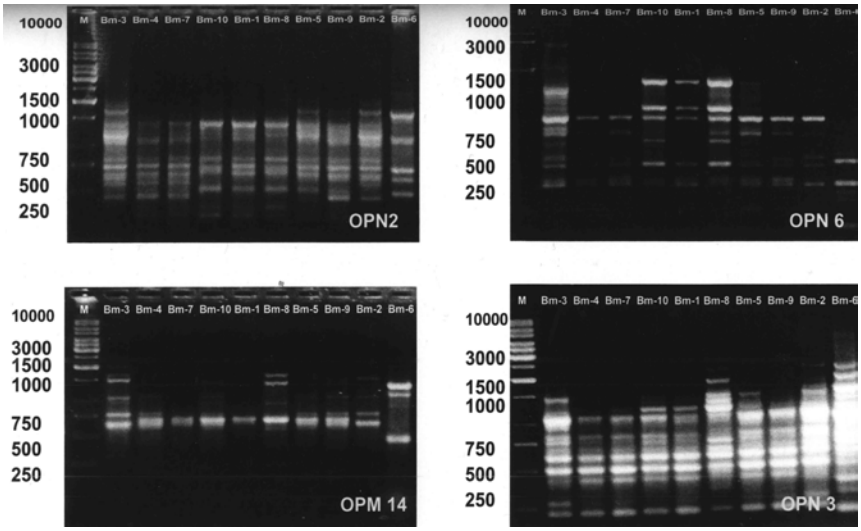

Fig. 2 Genetic polymorphism between various isolates of *Bipolarsis maydis* by RAPD_PCR using different random primers
Fig. 2 Polimorfismo genetico fra gli isolati di *Bipolaris maydis* determinato con RAPD_PCR usando differenti primers random

Genetic similarity of 58.7 per cent between Bm-1and Bm-9 isolates were closely behind them. Bm-2 isolate was genetically closely related with Bm-3 isolate (50.9 per cent). This isolate exhibited maximum divergence at genetic level from Bm-6 with dissimilarity of 64.4 per cent. It was clearly observed that Bm-1 and Bm-5 which had a common geographical origin were genetically closer. The isolates Bm-8 and Bm-10 from Udaipur exhibited higher similarity among them. Similarly the East Indian isolates, Bm-3 and Bm-2 had high genetic similarity. However, isolate Bm-6 was equidistant from rest of the isolates used in the present study (Tab. 1).

The similarity coefficients subjected to UPGMA-SAHN to produce a dendrogram, resulted in two major clusters (Fig. 3) which separated Bm-6 from rest of the isolates, which were grouped in two major cluster, one having Bm-3 and Bm-2 and the second one having the rest of the isolates in two sub clusters. The first sub cluster included Bm-4 and Bm-7 while Bm-1, Bm-5 and Bm-9 were grouped in second sub cluster. Isolates Bm-8 and Bm-10 were different from rest of the isolates in the second sub cluster thus remained one grouped.

It could be noted that there are two major clusters formed (Fig. 3). The prominent outcome of this clustering is that the East Indian isolates Bm-2 and Bm-3 were clustered in a separated sub group while Udaipur isolate including Bm-1 and Bm-5 were clustered in one sub group, the second cluster branched out into one cluster. The Bm-6 isolate was separate from rest of this isolates.

Several primers were observed to be good markers for some isolates of B. maydis in the present study. The banding pattern by OPM14 primer distinguished the isolate Bm-6 and isolate Bm-7 and Bm-1 isolate from rest of the isolates (Fig. 2).

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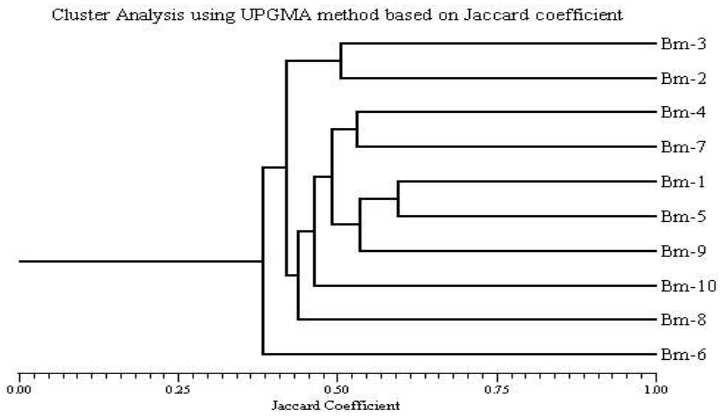

Fig. 3 Dendrogram showing relationship between different isolates of *Bipolaris maydis* based on RAPD analysis using UPGMA
Fig. 3 Dendrogramma degli isolati di *Bipolaris maydis* basato su analisi RAPD usando il metodo UPGMA

Conclusions

Random amplified polymorphic DNA (RAPD) has increasingly been used for the determination of polygenetic relationships and for the estimation of genetic diversity within fungal population. More recently the use of the molecular markers has given a boost to the analysis of accurate variation among various isolates of the pathogen.

BAKONYI et al. (1994) reported that species of *Bipolaris* formed a compact group, while *Drechslera* showed a strong dissimilarity; species of *Exserohilum* were scattered within the latter. SILVAKUMAR et al. (1995) reported that strains of *Corynespora* could be distinguished from a member of the closely related genus *Helminthosporum* on the basis of amplified ITS fragment size, but could not be typed individually as the ITS regions of all isolates exhibited identical size and restriction endonuclease digestion pattern.

ABADI et al. (1996) estimated DNA variability between thirteen isolates of *Exserohilum turcicum* races 0, 1, 2, 23 and N by RAPD and suggested that race 0, 1, and N may be grouped separately from race 23. BAKONYI et al. (1995) reported that *Drechslera* formed a large heterologus group, while species of *Bipolaris* and *Exserohilum* were more closely related. PELTONEN et al. (1996) reported that all *Drechslera teres* isolates could not be according to geographic origin, aggressiveness, growth rate or morphological features, indicating that the primers used in this study were natural markers for these characters. The primers were however, able to differentiate between isolates of *Helminthosporium* species. NICHOLSON et al. (1993) compared isolates of *B. maydis* from China (Races O, C, and T) using two techniques RAPD analysis indicated that race O and C isolates were more similar to each other than that of the race T isolates.

The selection of primers has great significance in pathotype identification and characterization and molecular mapping of the genome. These primers can also serve as a reference for integral comparison in future studies. In the presents study, characterization of *B. maydis* isolates by RAPD has proved useful in separating all the isolates from each other. It has also provided with primer marker that can be used to separate and distinguish each isolate. Unique band of 500 kb size by Bm-6 obtained by amplification with OPM 14 primer can be used further for development of SCAR marker. This possibility of distinguishing different isolates by rather simpler technique of genomic fingerprinting based on PCR-RAPD, could be of great important for use in epidemiological , taxonomical and evolutionary aspects.

In present investigations though no correlation could be found between morphological and genetical attributes, significant correlation between the genetic similarity and geographical origins could be

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established. These findings are similar to those obtained by FABRE et al. (1995) in a study on *Colletotrichum lindemuthianum* from different countries, they investigated grouping by DNA polymorphism in relation to the geographical origins of the isolates were formed two groups with isolates from Latin America occurring in both groups.

RAPD analysis offers the possibility of creating polymorphism without any prior knowledge of the DNA sequences of test organism. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species, if sufficient numbers of primers are screened. The method is also an economic technique for screening large number of samples. Although, some researchers are critical of the poor reproducibility of RAPD pattern, in present investigation, it was found that once the optimal RAPD conditions for a given species are established, the method works well for fungal samples even on crude DNA extracts.

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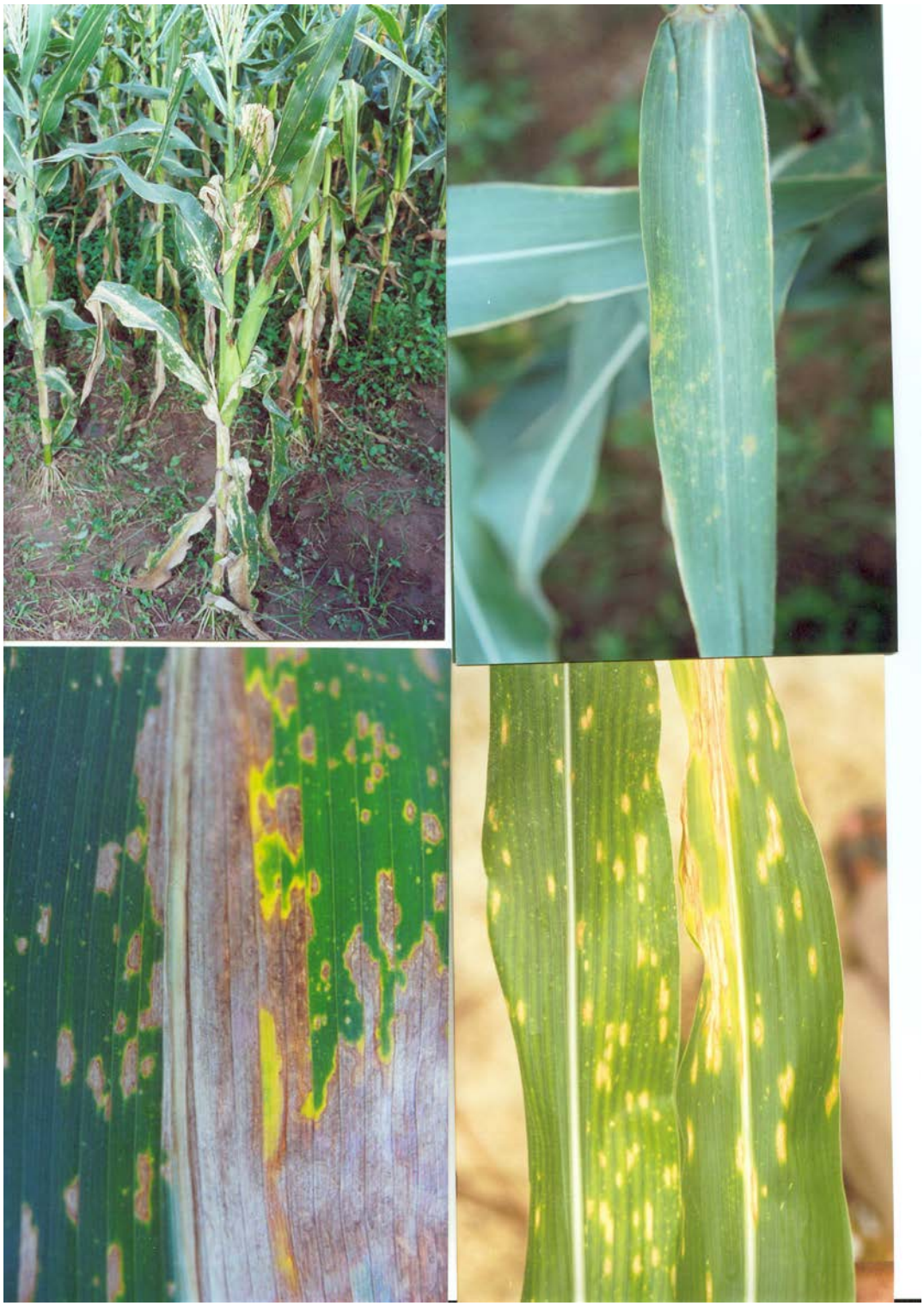

Fig. 4. Symptoms of maydis leaf blight caused by *Bipolaris maydis*
Fig. 4 Sintomi del seccume fogliare causato da *Bipolaris maydis*