

EXPLORATION OF MOLECULAR VARIABILITY IN *RHIZOCTONIA BATATICOLA*, THE INCITANT OF ROOT ROT DISEASE OF PULSE CROPS

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Abstract: The present study explored pathogenic and genetic variability among the eleven isolates of *Rhizoctonia bataticola* (Taub.) Butler from different pulse crops. Based on morphological characters, 11 isolates were categorized into three groups *viz.*, linear, fluffy, and linear at the end with fluffy growth at the center. Isolates also showed variability in sclerotial characters (intensity and shape) and intensity of pigment synthesis. All isolates were more aggressive on the original host from which it was isolated, which was shown by the variability in pathogenic characters. RAPD-PCR analysis has shown that genetic clustering agreed with the above findings in dendrogram analysis (2 clusters A and B). The black gram root isolates showed a maximum genetic similarity of 73 % with soya bean shoot isolate. Red gram shoot isolate showed 61% genetic similarity with green gram isolates. The findings from this study confirm the variability in *R. bataticola* isolates from pulses, according to their pathological as well as genetic characters. In the future, variability in pathogens will determine effective management practices.

Key words: genetic variability, pathogenic characters, phylogeny, pulses, RAPD-PCR, *Rhizoctonia bataticola*

INTRODUCTION

Rhizoctonia bataticola (Taub.) Butler (= *Sclerotium bataticola* Taub.) (Pycnidial stage: *Macrophomina phaseolina*) is a diverse omnipresent soil-borne fungal pathogen, infecting more than 500 plant species. The pathogen causes different types of diseases *viz.*, seedling blight, root rot, charcoal rot, wilt, stalk rot, stem blight, fruit rot, seedling decay and leaf blight in crop plants (Dhingra and Sinclair 1978). *R. bataticola* causes up to 60% yield loss. Different isolates of *R. bataticola*, obtained from different plant species, and plant parts of the same host showed variability (Prameela and Singh 1998; Meena *et al.* 2006). Sixty four isolates of *M. phaseolina* from sunflower (Manici *et al.* 1992) and cotton (Monga *et al.* 2004) fell into 3 groups *viz.*, highly virulent, virulent and poorly virulent, of pathogenic variability. Perhaps, evaluation of the genetic diversity in pathogen isolates has been an initial step towards understanding the population structure. Molecular techniques have become reliable and are highly suitable tools for identifying pathogen species and for assessing genetic variation within collections and populations. RAPD offers a promising, versatile and informative molecular tool to detect genetic variation within populations of plant pathogens (Chiocchetti *et al.* 1999). Random amplified polymorphic DNA analyses have been used to characterize genetic diversity of different isolates of *M. phaseolina* (Almeida *et al.* 2003). Variability in molecular characters is used for determining resistant cultivars (Thirumalaisamy

et al. 2006) and for the evaluation of the germplasm resistant line (Shekhar *et al.* 2006).

Hence, the present investigation was meant to detect the variability among the different isolates of *R. bataticola* from roots, leaf, and seeds of pulse crops (blackgram, greengram, cowpea, soybean, and redgram) based on morphological, pathogenic and genetic characters.

MATERIALS AND METHODS

Isolation of *R. bataticola* isolates

Pulse crops (red gram, greengram, cowpea, soybean, blackgram) showing typical root rot symptoms were collected from the Tamil Nadu Agricultural University Research Farm. The infected portion collected from roots, shoots and seeds were surface sterilized with 0.1% mercuric chloride for 30 sec, washed subsequently in three changes of sterile distilled water, and placed on Potato dextrose agar (PDA) medium. It was purified by the single hyphal tip method. Pure culture of the different isolates of *R. bataticola* was maintained on PDA slants for further studies.

Mass multiplication of *R. bataticola* inoculum

The isolates of the fungus were multiplied in sand maize medium (Riker and Riker 1936). Sand and ground maize seeds were mixed in a ratio of 19:1 moistened to

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50 % moisture content, filled in polypropylene bags, and autoclaved at 20 psi for two hours. Four actively growing mycelial discs (9 mm) of the pathogen isolates were inoculated into each polypropylene bags under aseptic condition. The polypropylene bags were then incubated at room temperature (28±2°C) for 15 days, and the inoculum thus obtained was used for the experiments.

Morphological studies

Growth pattern

The individual *R. bataticola* isolates of blackgram (shoot, root and seed) as well as shoot and root isolates from greengram, cowpea, soybean and redgram were grown separately in PDA and the growth pattern was studied at a room temperature of 28±2°C. The colony color, growth of colony, and colony characters were measured 3 DAI (Days after inoculation). Three replications were done.

Sclerotial number

Three 9 mm diameter disc containing the sclerotia of *R. bataticola* (11 isolates) were punched from each Petri plate. These discs were placed in a beaker containing 10 ml of sterile distilled water and stirred for 30 min. to separate the sclerotia from the medium. The entire contents were squeezed through cheese cloth, washed in several changes of distilled water, and transferred to a glass vial containing 2.5 ml of 2.5% ammonium sulphate. The sclerotia, which floated after 10 min., were filtered through Whatman No. 42 filter paper and rinsed with the distilled water. The filter paper with these sclerotia was removed and the number of sclerotia counted with a stereo binocular microscope.

Sclerotial size

For each isolate, 100 sclerotia were collected at random and their size was measured using an ocular micrometer in a calibrated microscope. The shape and intensity of sclerotia produced was also recorded.

Pigment production

The pigment synthesis of pathogen was observed at regular intervals in peptone sucrose broth, and the intensity (+ – low, ++ – medium and +++ – high) was recorded.

Assessing the virulence of *R. bataticola* isolates

The potting mixture was prepared by thoroughly mixing clay loam soil, sand and farm yard manure at a 1:1:1 ratio. The inocula of each isolate of *R. bataticola* collected from different parts of pulse crops were separately mixed at 100 g/kg (w/w) with the unsterilized soil, filled in 30 cm earthen pots ten days before sowing. Surface sterilized (using 0.1% MgCl₂ solution for 30 sec. followed by two washings in sterile water) blackgram, greengram, cowpea, soybean, redgram seeds were sown – 10 seeds per pot. Three replications were maintained in a completely randomized design. The pots were kept in a glasshouse with regular, judicious and uniform watering. The root rot incidence was recorded 60 DAS (Days after sowing). The Percent disease incidence (PDI) was calculated as below:

$$P = \frac{\text{Total number of plants infected}}{\text{Total number of plants observed}} \times 100$$

RAPD-PCR analysis for assessing the variation among the different isolates

The primers used for molecular analyses were OPA-01 (CAGGCCCTTC), OPA-02 (TGCCGAGCTG), OPG-03 (GAGCCCTCCA), OPA-08 (GTGACGTAGG), OPA-09 (GGGTAACGCC), OPA-11 (CAATCGCCGT), Primer 1(AAGAGCCCGT), OPU 7 (CCTGCTCATC), OPA13 (CAGCACCCAC) and OPP 14 (CCAGCCGAAC) obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. Amplification reactions with a total volume of 20 µl was standardized as follows for taxonomic analysis of RAPDs: 0.5 to 5 ng of template DNA (in 10 µl of H₂O), 0.6 mM primer, 50 mM each dNTP, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), and 0.5 U of Taq DNA polymerase. In each amplification reaction, a control sample without DNA was included. Samples were quickly transferred in Mastercycler gradient (Eppendorf, Germany), preheated at 96°C, and incubated at this temperature for 3 min. to denature the DNA completely. This was followed by 38 cycles of amplification consisting of 45 sec at 95°C to denature the DNA, 1.5 min. at 40°C to anneal the primers, and 2 min. at 72°C to extend the annealed primers. A final extension step of 10 min. was programmed to ensure complete extension of the amplified products. The amplified fragments were analyzed by electrophoresis of 10 µl of the amplification reaction mixture in 2% agarose gels run in 1x Tris Borate EDTA (TBE) buffer.

RAPD Data analysis

The band patterns were scored for RAPD primers in each of the isolates starting from the small sized fragments to the large sized ones. Presence and absence of each band in each isolate was coded as 1 and 0 respectively. The scores were used to create a data matrix to analyze the genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA). A dendrogram was constructed based on Jaccard's similarity coefficient using the marker data from eleven *R. bataticola* isolates with unweighted pair group method (UPGMA).

RESULTS

Variability in morphology characters

Eleven isolates of *R. bataticola* from different parts of the pulse crops have shown morphological variability, including sclerotial character and pigment productions. In the shoot isolates of blackgram, cowpea, soybean and greengram a light grey colony color was assigned. The root isolates of blackgram and cowpea were assigned a dark grey colony color (Table 1). The seed isolate of blackgram and root isolate of greengram and soybean were assigned a light grey colony with a whitish center (I_c). The growth patterns of the eleven isolates of *R. bataticola* were studied on PDA medium and were found to differ among themselves in growth pattern. Based on the different growth patterns, the isolates were categorized into three groups

such as linear (root isolates of blackgram, greengram, redgram and greengram shoot isolate), fluffy (shoot isolate of blackgram, redgram and soybean) and linear at the end with fluffy growth at center (blackgram seed isolate cowpea and soybean root isolate). Observations regarding the growth rate were also done and the isolates were categorized into three groups, fast (blackgram seed isolate and shoot and root isolates of redgram and root isolates of cowpea), moderate (cowpea shoot isolate, greengram and blackgram shoot and root isolates) and slow growing (soybean shoot and root isolate).

Variability in sclerotia formation

The days taken for the formation of sclerotia were also recorded and it was observed that the shoot and root isolates of soybean took respectively 5.3 and 5.6 days for sclerotial formation. The other isolates recorded 2.3 to 4.0 days for the formation of sclerotia. The intensity of sclerotia formation was observed in all the eleven isolates. It was observed that the root isolates of blackgram, cowpea, greengram, and redgram produced abundant sclerotia whereas moderate sclerotial formation was observed in shoot isolates of blackgram, cowpea, greengram and redgram. The seed isolate of blackgram and root isolate of soybean also recorded moderate sclerotial formation while the shoot isolate of soybean produced less sclerotia. Among the isolates, the diameter of sclerotia ranged from 43.6 μm to 89.9 μm . The sclerotia of blackgram root isolate recorded a maximum diameter of 89.9 μm and were

significantly superior and were followed by redgram root isolate (86.85 μm). The sclerotia of the shoot and root isolates of soybean was the smallest at 43.6 and 43.8 μm in diameter, respectively, and were on par with one another. The shape of the sclerotia was categorized into two groups *viz.*, round and irregularly oblong. The blackgram and redgram shoot recorded round sclerotia while all the other isolates recorded irregularly oblong sclerotia (Table 1).

Pigmentation

It was interesting to note that the eleven isolates of *R. bataticola* inoculated in the peptone sucrose liquid broth, synthesized pink pigment. The blackgram shoot isolate recorded low pink pigment intensity while intensity was moderate in cowpea, greengram, soybean shoot and root isolates and in redgram root isolate. High pigmentation was observed in blackgram root, seed and redgram shoot isolate (Table 1).

Variability in pathogenic characters

The *R. bataticola* isolates from various plant parts differed in their pathogenicity. The blackgram shoot and root isolate was highly virulent and significantly superior against blackgram recording 90.60 and 88.58%, respectively of root rot incidence. The next best susceptible host was greengram. It should be noted that the seed isolate was less virulent on its original host. The seed isolate recorded only 58.00% root rot incidence on blackgram and it was followed by greengram (56.28%) (Fig. 1A).

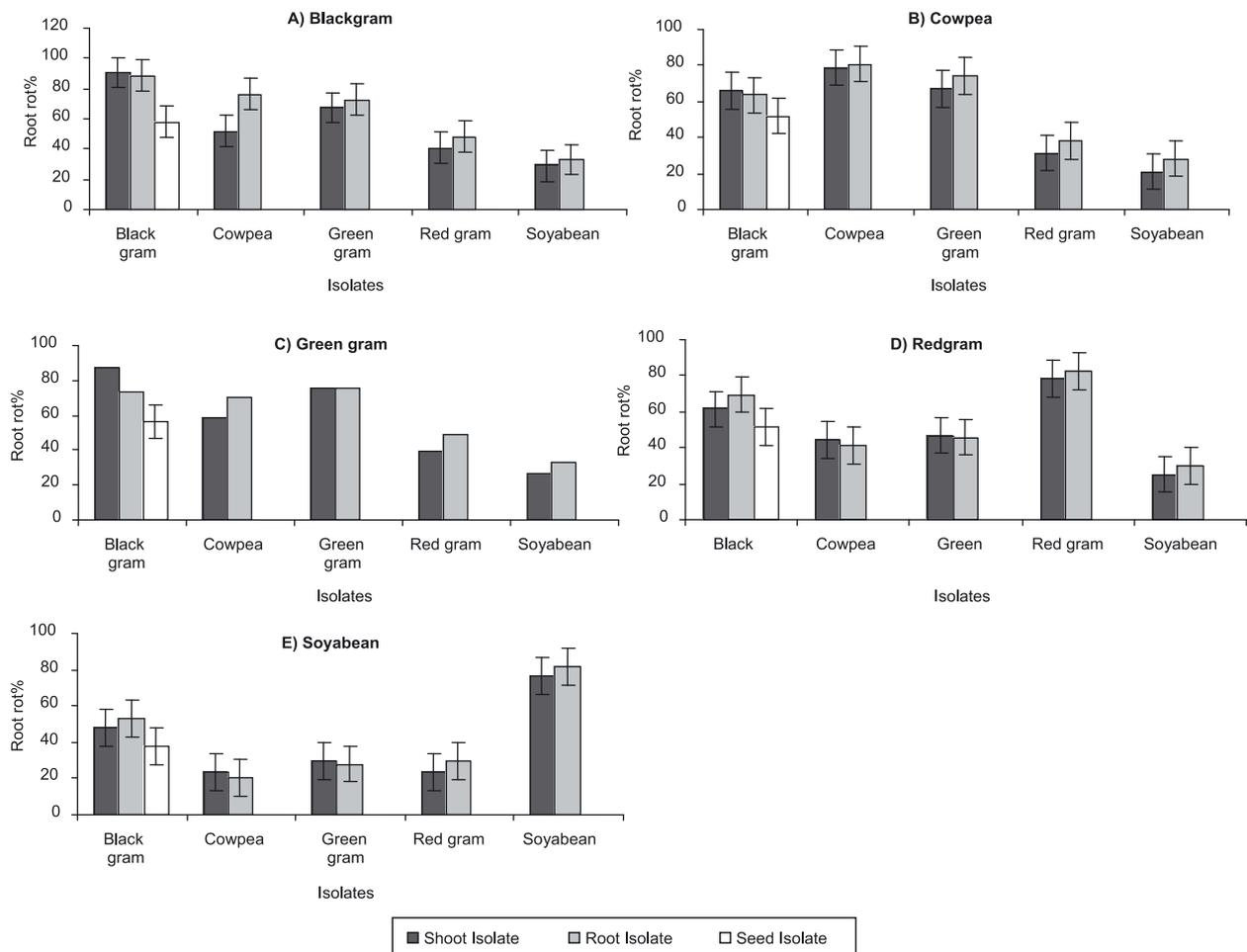


Fig. 1. Pathogenic variability in *R. bataticola*

The cowpea isolate recorded a maximum of 80.87% root rot incidence on cowpea and it was significantly superior. This was followed by blackgram (76.51%) and greengram (70.0%). Soybean recorded the least incidence of 20.28% root rot incidence. The results confirmed that the pathogen is more aggressive on the original host in which it has been isolated, and root isolate is more virulent in inducing root rot compared to the shoot isolates (Fig. 1B).

The root isolate of greengram recorded 76% root rot incidence after 60 DAS and it was significantly superior and was followed by cowpea which recorded 74.25% root rot incidence (Fig. 1C). The isolates of greengram were not aggressive on soybean. The root isolate recorded only 28% root rot incidence on soybean 60 DAS, proving its less virulent nature on soybean.

The root isolate of red gram recorded the maximum of 82.47% root rot incidence in redgram and it was significantly superior (Fig. 1D). The soybean crop recorded the least incidence of 29.3% root rot against root isolate of redgram.

The root isolate of soybean recorded 81.6% root rot on soybean and it was individually significantly superior, revealing the compatibility of the host and its pathogen (Fig. 1E).

Variability in genetic characters

Isolates of *R. bataticola* formed two clusters A and B, at a similarity coefficient at level 0.58. Cluster A consisted of two sub-clusters viz., A₁ and A₂. Sub-cluster A₁ had two sub-groups viz., SA1-1, SA1-2. The isolate of blackgram shoot (I₁), blackgram root (I₂), blackgram seed (I₃),

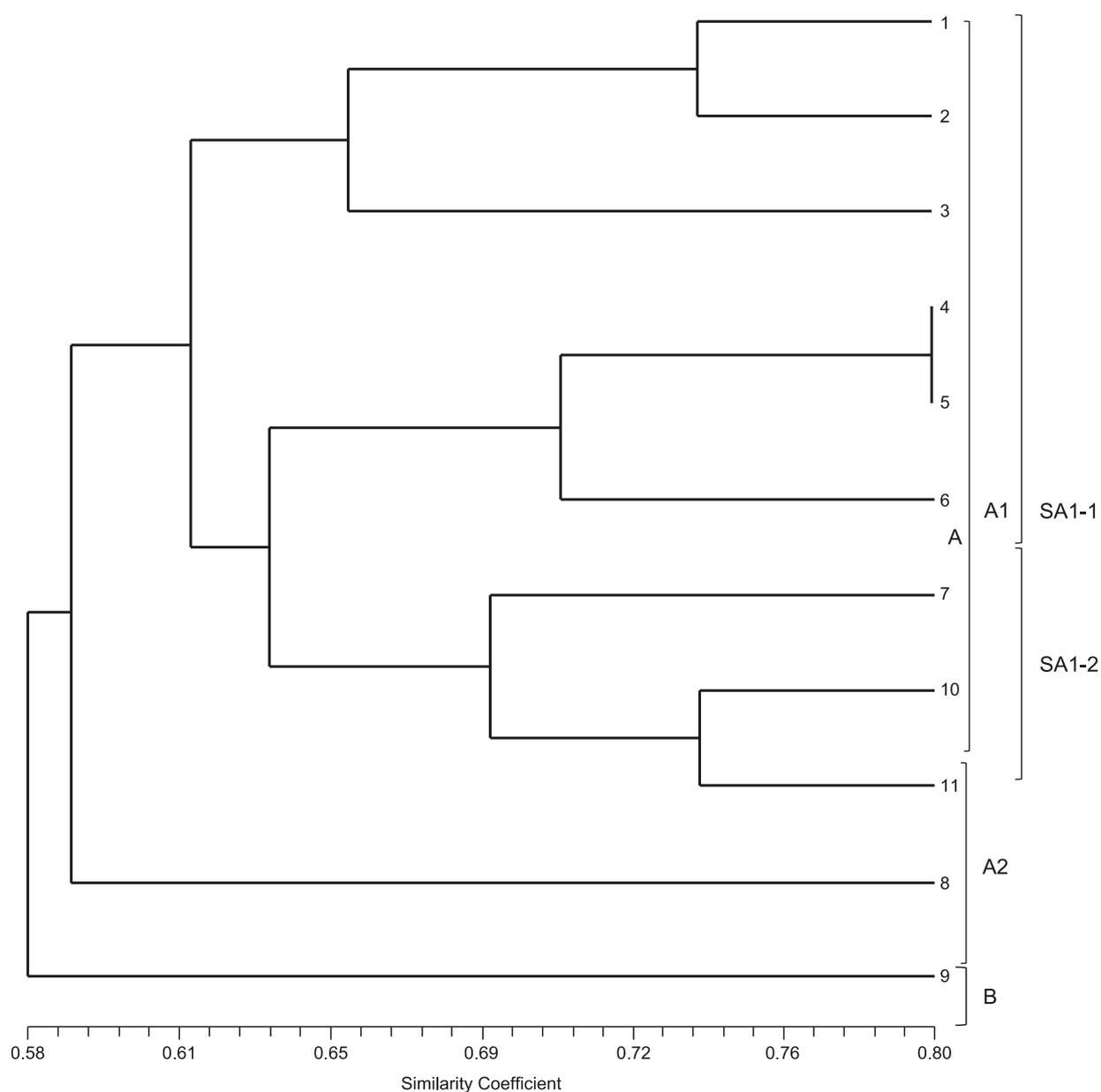


Fig. 2. Dendrogram showing the similarity and successive clustering of 11 isolates of *R. bataticola*

cowpea shoot (I_4), cowpea root (I_5), greengram shoot (I_6), greengram root (I_7), soybean shoot (I_{10}), soybean root (I_{11}), and redgram shoot (I_8) were grouped under cluster A_1 . Blackgram shoot (I_1), blackgram root (I_2) and blackgram seed (I_3) were grouped under the first sub-group of A_1 . Cowpea shoot (I_4), cowpea root (I_5), greengram shoot (I_6), greengram root (I_7), soybean shoot (I_{10}) and soybean root (I_{11}) were grouped under the second sub-group of A_1 . The cowpea shoot (I_4) and cowpea root (I_5) shared 100 per cent genetic similarity with each other. Isolates I_4 and I_5 showed an 80 % similarity. The redgram root (I_9) alone was grouped under cluster B (Fig. 2).

DISCUSSION

This study has shown that there is considerable variability among *R. bataticola* isolates obtained from infected pulse crops. Based on the morphological characters, isolates were grouped into three groups such as linear, fluffy and linear growth with fluffy mycelial growth at centre. The rate of mycelial growth was also categorized as fast, moderate and slow growth. Previous investigations showed that, based on mycelium characters, seven isolates of *R. bataticola* were put in groups viz: black in centre and white in periphery, grayish, charcoal black, fluffy whitish with black periphery and submerged (Sobti and Sharma 1992). Sharma *et al.* (2004) observed the fluffy, fast –growing, dull-white growth of *Macrophomina phaseolina* from pearl millet. Sesame isolate as well as horse gram isolate recorded flat, compact white to black growth, while mothbean isolate exhibited fluffy growth with a black centre.

In addition to this, the degree of production of sclerotia was found to be useful for direct correlation between sclerotial production and pathogenicity of isolates. The intensity of sclerotia formed in all the eleven isolates varied as well as their size. As for the sclerotial formation, the isolates were grouped as abundant, moderate and less sclerotia forming isolates. Jain *et al.* (1973), reported that the size of the sclerotia was maximum in the stem and soil isolates. Byadgi and Hegde 1985, reported that Gliricidia isolate of *R. bataticola* produced the biggest sclerotia with a mean diameter of 101.51 μ m while cowpea isolate produced the smallest sclerotia (66.88 μ m). *R. bataticola* sclerotia size varied from 58.83 μ to 126.63 μ (Monga and Raj 1994). From the literature, and from the present study it is clear that variability exists in *R. bataticola* sclerotia.

M. phaseolina isolates from onion, carnation, olive and chickpea produced a dense red color and the pigment production was the best on potato yeast extract agar (Ullukus 1984). The results presented here showed that eleven isolates of *R. bataticola* synthesized pink pigment in the peptone sucrose liquid broth, and their intensity varied among the isolates.

The experiment was carried out to identify the pathogenic variability of isolates. It was interesting to note that all the isolates were aggressive in the respective, original host in which it was isolated. Among the blackgram shoot, root and seed isolate, the root isolate recorded higher incidence of root rot when compared to shoot and seed isolate. The seed isolate recorded only a 58% root rot

incidence on blackgram. The same trend was observed on all the other pulse hosts. Hence the results revealed that there was considerable variation in the pathogenicity of the eleven isolates on the different pulse host. There are differing reports in the literature on the pathogenic properties among the isolates of *R. bataticola* (Latha *et al.* 2002).

RAPD-PCR has been successfully used to identify strains and races in phytopathogenic fungi (Williams *et al.* 1990). It has also been used for studying inter- and intraspecific variability among populations from different as well as from the same geographic regions. The RAPD pattern analysis showed variations at the DNA level, and is thus suitable for differentiation of *M. phaseolina* isolates below species level (Franco *et al.* 2006). Using PCR, closely related strains of a pathogen can be distinguished without prior knowledge of the nature of polymorphic regions by the use of RAPD. PCR-based DNA fingerprinting, particularly with short oligonucleotide primers, has been used by various researchers for the analysis of genetic variation in plant pathogens (Purkayastha *et al.* 2006). The results presented here show that, in dendrogram analysis, two clusters were formed (A and B). Cluster A consists of blackgram root, shoot, seed isolates, soybean, root and shoot isolates, cowpea root and shoot isolates, greengram root and shoot isolates and redgram shoot isolate. Red gram root isolate alone, was grouped under cluster B.

Several people have researched the genetic variability of *M. phaseolina* to work out the pathogenic and genetic patterns of diversity and genetic specialization (Jain *et al.* 1973; Almeida *et al.* 2003; Janar *et al.* 2003). Further, fine tuned characterization of root rot pathogen *R. bataticola*, and more improvement in pulse root rot resistance and varietal development strategies needs to be done.

Finally, the experimental research such as morphological, pathological and genetic variability in *R. bataticola* isolates from pulse crops, significantly determines the effective, future management practices.

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POLISH SUMMARY

BADANIA NAD MOLEKULARNĄ ZMIENNOŚCIĄ GRZYBA *RHIZOCTONIA BATATICOLA*, CZYNNIKA SPRAWCZEGO ZGNILIZNY KORZENI ROŚLIN MOTYLKOWATYCH

Badano patogeniczność i genetyczną zmienność 11 izolatów grzyba *Rhizoctonia bataticola* (Taub.) Butler pochodzących z różnych upraw roślin motylkowatych. Biorąc pod uwagę cechy morfologiczne 11 izolatów patogena sklasyfikowano je w trzech grupach: o liniowym wzroście, puszystym oraz liniowym zakończonym puszystym centrum wzrostu. Izobaty grzyba wykazywały także różnice w procesie wytwarzania sklerot (częstotliwość i kształt), a także różniły się intensywnością syntezy pigmentu. Wszystkie badane izobaty były bardziej agresywne w stosunku do roślin gospodarzy, z których zostały pierwotnie wyizolowane, co potwierdza zmienność cech patogeniczności. Wyniki analizy RAPD-PCR wykazały, że klastrowanie genów było zgodne z dendrogramem wyżej wymienionych cech (2 klastry A i B). Izobaty patogena pochodzące z korzeni fasoli mango wykazywały najsilniejsze genetyczne podobieństwo rzędu 73% do izolatów z łodyg fasoli sojowej. Natomiast izolat patogena z nikli indyjskiej wykazał 61% genetycznego podobieństwa do izolatów pochodzących z roślin fasoli złotej. Wyniki prezentowanych badań potwierdziły występowanie zmienności izolatów grzyba *R. bataticola* pochodzących z upraw roślin motylkowatych w zależności od ich cech genetycznych i patogeniczności. Poznanie zmienności patogenów pozwoli w praktyce na wybór efektywniejszych metod ich zwalczania.