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# MOLECULAR DETECTION, TRANSMISSION AND HISTO-PATHOLOGICAL STUDIES OF SEED-BORNE FUNGAL INFECTION OF SOYBEAN (*GLYCINE MAX* (L.) MERILL)

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**ABSTRACT**
Soybean crop being an oilseed crop has been receiving wider attention in India. Over hundred pathogens are known to affect soybean, of which majority are associated with soybean seeds. Seed borne infection of soybean is very important discouraging factor, which possess a serious problem in seed certification. Seed borne infection of the pathogen is generally detected by conventional methods such as blotter method and agar plate method followed by microscopy. But, these techniques are time consuming and not sensitive. The early detection of seed borne infection by novel molecular techniques like Polymeric Chain Reaction (PCR) using fungal specific primer has tested here. ITS1 and ITS4 were used for the diagnostic purpose of seed-borne fungal infection. Oligonucleotide specific primers targeting the ITS region have been demonstrated to selectively detect several agriculturally important fungi. Further the amplified product sequenced and revealed the sample genome has homology with *Cercospora kikuchii* and *Rhizoctonia bataticola* genome. The pathogenic ability of seed borne fungi was proved in transmission studies carried out by seedling symptom test and pot culture studies. Finally the histopathological studies revealed the location of seed borne fungi in the infected soybean seeds. In most of the observations the fungal pathogens were occupied in the pericarp, hilum and endosperm regions.

## INTRODUCTION

Seed borne infection or infected seed is very important discouraging factor, which possess a serious problem in seed certification. Although infected seeds which may otherwise be viable with prescribed germinability as per certification standards, many times it may not be acceptable as seed because of poor physical appearance, high expected incidence of seed born fungi and mycotoxin such as aflatoxin. Soybean *Glycine max* (L.) Merill is a native of eastern Asia popularly known as 'Chinese Pea' further it is also referred to as wonder crop of the 20th century because of its characters and usage The infection of soybean (*Glycine max*) seed caused by various fungal pathogen viz. purple seed stain(*Cercospora kikuchii* (Matsumoto and Tomoy)), anthracnose (*Colletotrichum truncatum* (Schw.) Andrus and Moore ), pod and stem blight (*Phomopsis* spp.*) and Fusarium* seed infection on soybean seeds, long used for diagnostic purposes, is now being used as indicative of quality (Sinclair and Backman, 1989) . But mere external appearance will not give reliable information about the internal seed infection as well as latent infection. Due to this, presently more focus is giving to the molecular detection of the seed-borne infection which is more reliable and rapid approach (Molouba *et al*., 2001) along with histopathological and transmission studies,

Oligonucleotide specific primers or probes targeting the ITS region have been demonstrated to selectively detect several agriculturally important fungi like *Trichoderma*, *Hypocrea* (Irina *et al*., 2005). Bandamaravuri *et al*. (2007) developed a rapid diagnostic test that can exactly identify and detect *M. phaseolina* isolates, both *in vitro* and *in vivo* conditions. Nuclear rDNAs, particularly in the internal transcribed spacer (ITS) region, are good targets for phylogenetic analysis in fungi. They developed specific primers and oligonucleotide probe (within the ITS region) and subsequent evaluation of their efficiency for identification/detection of *M. phaseolina* under in vitro conditions.Molecular diagnosis of soybean seed-borne fungal infection using fungal specific primer is reliable one and early detection is possible.

Here another attempt was made to study the fungal seed transmission along with different inoculum density. Ellis *et al*. (2011) conducted a study on *Fusarium graminearum* causes seed decay and damping-off of soybean. This study evaluated the effect of inoculum density of *F. graminearum*, temperature, and fungicide seed treatments on disease development. The histopathological studies of *Macrophomina* infected soybean seeds revealed that the fungus lies as hyphae and sclerotia in all the three layers (palisade, hypodermis and aleurone) of seeds coat as inter and intra- cellular mycelium and as sclerotia in cotyledons of infected seeds. The fungus transmits from seed to seedling in a non-systemic manner (Arya *et al*., 2004).

Presently little information is available on molecular detection

of seed borne fungal infection of soybean using PCR through fungal specific primer, transmission of seed-borne pathogen from seed to plant and their histopathology. Keeping these factors in view and considering their importance in present scenario of seed production, seed industry and quarantine, present investigation was undertaken.

## MATERIALS AND METHODS

### Molecular diagnosis of seed-borne fungal infection of soybean through PCR using fungal specific primer

### DNA isolation from healthy and naturally infected soybean seed

DNA was extracted from healthy as well as naturally infected seed samples by using CTAB method (Doyle and Doyle 1990). Cetyl trimethyl ammonium bromide (CTAB) was used along with other reagents to liberate nucleic acids from the plant cell. This is an efficient method for isolating genomic DNA. The high molecular weight DNA obtained was purified by phenol: chloroform method to remove the proteins and other plant debris. Materials used for DNA extraction are extraction buffer - 4% CTAB, chloroform: isoamylalcohol mix (24:1), isopropanol, 70% alcohol, TBE buffer.The infected or apparently healthy seeds and uninfected healthy seeds were washed in water and the excess water was blotted with blotting paper and air dried briefly. Two gram seed material was weighed from each infected and control sample. The seed tissues were ground well to powder form using liquid nitrogen. Powdered seed tissues were transferred to the sterilized micro-centrifuge tube. Hot extraction buffer was added (CTAB) to each tube @ 750$\mu$L and 10$\mu$L of b-mercaptoethanol. The tubes were incubated at 65ºC in a water bath for 15-20 min with gentle inversion. Equal volume of chloroform: isoamylalcohol (24:1) was added and mixed well by inverting the tubes then contents were centrifuged at 6000 rpm for 20 minutes at 10$^0$C. The supernatant was collected and equal volume of chilled Isopropanol was added and was kept overnight at -70ºC and again centrifuge the tube at 6000 rpm for 20 minutes at 10ºC. Decant the supernatant and washed the pellets with 70% alcohol. Centrifuge the tube at 6000 rpm for 20 minutes at 10ºC. Decant the centrifuge and the pellet was air dried until alcohol smell disappeared and sterile water or TE buffer was added to the air dried pellet and stored at -20ºC.

From each DNA samples isolated by the above methods, 2$\mu$L of DNA was taken and diluted 1:50 in millpore water. Absorbance was recorded thrice at 260 nm as well as at 280 nm in a UV spectrophotometer. DNA concentration was measured at 260 nm, and DNA yield was calculated by multiplying the mean concentration and hydration volume and quality of genomic DNA was checked by Agarose gel (0.8 %) electrophoresis.

### PCR amplification

Polymerase chain reaction (PCR) is the in-vitro, primer-directed, enzymatic amplification of nucleic acids (Erlich *et al.,* 1988;

**Table 1: Effect of different seed inoculum levels of *Rhizoctonia bataticola* on per cent seed infection and germination**

| Sl. No. | Treatments | Per cent infection | Per cent germination |
|---|---|---|---|
| 1 | $T_1$- Seeds soaked in $4\times10^6$ propagules/10 mL | 100.00(90.05)* | 0.00(7.27) |
| 2 | $T_2$-Seeds soaked in $4\times10^5$propagules /10 mL | 100.00(90.05) | 0.00(7.27) |
| 3 | $T_3$- Seeds soaked in $4\times10^4$propagules /10 mL | 73.33(58.94) | 43.33(41.19) |
| 4 | $T_4$-Seeds soaked in $4\times10^3$propagules /10 mL | 63.33(52.76) | 56.67(48.86) |
| 5 | $T_5$- Un-inoculated (Apparently healthy seeds) | 23.33(28.90) | 76.67(61.15) |
| | SEm $\pm$ | 1.65 | 1.28 |
| | CD at 1% | 7.84 | 6.07 |

*Figures in parentheses indicate arcsine values

**Table 2: Effect of different seed inoculum levels of *Cercospora kikuchii* on per cent seed infection and germination**

| Sl. No. | Treatments | Per cent infection | Per cent germination |
|---|---|---|---|
| 1 | $T_1$- Seeds soaked in $3\times10^6$propagules /10 mL | 100.00(90.05)* | 0.00(7.27) |
| 2 | $T_2$-Seeds soaked in $3\times10^5$propagules /10 mL | 80.00(63.47) | 36.67(37.29) |
| 3 | $T_3$- Seeds soaked in $3\times10^4$propagules /10 mL | 66.67(54.77) | 53.33(46.93) |
| 4 | $T_4$-Seeds soaked in $3\times10^3$propagules /10 mL | 46.67(43.11) | 60.00(50.79) |
| 5 | $T_5$- Un-inoculated (Apparently healthy seeds) | 16.67(24.11) | 83.33(65.94) |
| | SEm $\pm$ | 1.71 | 1.71 |
| | CD at 1% | 8.12 | 8.12 |

*Figures in parentheses indicate arcsine values

**Table 3: Effect of different seed inoculum levels of *Colletotrichum truncatum* on per cent seed infection and germination**

| Sl No | Treatments | Per cent infection | Per cent germination |
|---|---|---|---|
| 1 | $T_1$- Seeds soaked in $5.5\times10^6$propagules /10 mL | 100.00(90.05)* | 0.00(7.27) |
| 2 | $T_2$-Seeds soaked in $5.5\times10^5$propagules /10 mL | 100.00(90.05) | 0.00(7.27) |
| 3 | $T_3$- Seeds soaked in $5.5\times10^4$propagules /10 mL | 73.33(58.94 | 43.33(41.19) |
| 4 | $T_4$-Seeds soaked in $5.5\times10^3$propagules /10 mL | 50.00(45.02) | 63.33(52.76) |
| 5 | $T_5$- Un-inoculated ( Apparently healthy seeds) | 13.33(21.42) | 86.67(68.62) |
| | SEm $\pm$ | 1.66 | 1.61 |
| | CD at 1% | 7.88 | 7.62 |

*Figures in parentheses indicate arcsine values

**Table 4: Effect of different seed inoculum levels of *F. oxysporum*on per cent seed infection and germination**

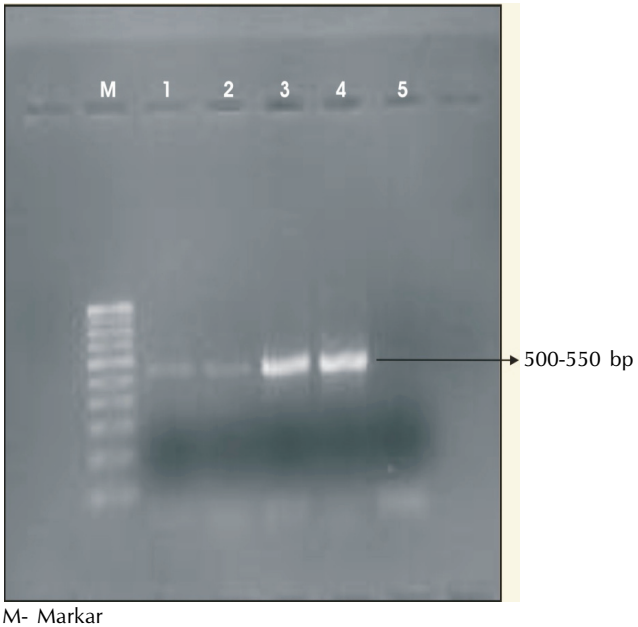
| Sl No | Treatments | Per cent infection | Per cent germination |
|---|---|---|---|
| 1 | $T_1$- Seeds soaked in $3.75\times10^6$propagules /10 mL | 100(90.05)* | 0.00(7.27) |
| 2 | $T_2$-Seeds soaked in $3.75\times10^5$propagules /10 mL | 100(90.05) | 0.00(7.27) |
| 3 | $T_3$- Seeds soaked in $3.75\times10^4$propagules /10 mL | 73.33(58.94) | 50.00(45.02) |
| 4 | $T_4$-Seeds soaked in $3.75\times10^3$propagules /10 mL | 56.67(48.86) | 53.00(46.74) |
| 5 | $T_5$- Un-inoculated (Apparently healthy seeds) | 23.33(28.90) | 76.67(61.15) |
| | SEm± | 1.78 | 1.23 |
| | CD at 1% | 8.44 | 5.83 |

*Figures in parentheses indicate arcsine values

**Table 5: Location of seed borne fungi in different seed parts of infected soybean seed by component plate plating technique**

| Sl. No. | Fungi observed | Seed parts tested Pericarp | Embryo | Endosperm | Hilum |
|---|---|---|---|---|---|
| 1 | *Rhizoctonia bataticola* | + | + | + | - |
| 2 | *Cercospora kikuchii* | + | - | + | + |
| 3 | *Fusarium oxysporum* | + | + | - | + |
| 4 | *Colletotrichum truncatum* | + | - | - | + |
| 5 | *Aspergillus flavus* | + | + | + | + |
| 6 | *Aspergillus niger* | + | + | - | - |

+ = Presence of pathogen; - = Absence of pathogen




M- Markar
1 and 2- *Cercospora kikuchii* infected seed
3 and 4- *Rhizoctonia baticola* infected seed
5- Healthy Seed

**Figure 1: Molecular diagnosis of soybean seed borne fungal infection through PCR**

Saiki *et al.,* 1988). DNA samples isolated from inoculated jute seeds as well as from naturally infected seeds were assessed by PCR using fungal specific primer pair and then used for PCR amplification using the primers in an Eppendorf Master Cycler at the optimal annealing temperature. Before that the annealing temperature of primers was standardized. Amplification was carried out in a total reaction volume of 20 $\mu$L with the components *viz.*,genomic DNA (100 ng), Taq assay buffer(10X), dNTPs (2mM), $MgCl_2$ (2.5mM), forward primer (ITS1-TCCGTAGGTGAACCTGCGG), reverse primer (ITS4-TCCTCCGCTTATTGATATGC) (Sharon *et al*., 2008) 10 picomoles /mL each and Taqpolymerase (5 units). The amplification was carried out using a gradient thermal cycler (Bio-Rad, CA, USA). The amplification profile was 5 min initial denaturation at 94ºC, denaturation at 94ºC for 1 min, annealing at 56ºC for 60 s and elongation at 72ºC for 1 min followed by 30 cycles and a final elongation at 72ºC for 10 min. PCR products were visualized in 16% agarose gel stained with ethidium bromide. The resulting amplified product was resolved on Agarose (1.0 %) gel to confirm the gene (Biswas *et al.,* 2012).

**Effect of different seed inoculum densities on disease incidence**

Seed to plant transmission studies were carried out under pot culture condition. Study was under taken by using apparently healthy seeds to know the effect of different seed inoculum densities and rapidity of disease incidence. *Rhizoctonia bataticola* (Taub.) Butler, *Cercospora kikuchii* (Matsumoto and Tomoyasu), *Colletotrichum truncatum* (Schwein.) Andrus and W.D.Mooreand *Fusarium spp.*, isolated from naturally infected soybean seeds, was cultured on PDA, at 20 ± 2ºC for one week. The fungal propagules (mycelial bits) were isolated by taking a 0.5 cm disc using a cork borer. The discs were gently mixed with 5ml of sterile water by using a magnetic stirrer and propagules were adjusted to required concentration by using haemocytometer.

No of propagules in the diluted suspension/milliliter = Average no of propagules above one large square × 1mL / 0.004 $mm^3$ (or)

No of propagules in the diluted suspension/milliliter = No of propagules counted × 250,000

This number has to be multiplied by the original dilution of the suspension to ascertain the density of original suspension in number of cells /mL (Karuna Vishunavat and Kolte, 2005).

Apparently healthy seeds of soybean cultivars were surface sterilized for 3 min 0.5 % sodium hypochlorite solution, and washed in sterile distilled water. The sterilized seeds were soaked in different inoculum level under vacuum for 30

minutes, and dried at room temperature overnight. The seeds of control treatments were similarly treated except that they were soaked in sterile distilled water. Five seeds were sown in each pot and replicated 4 times in each treatment. The pots were incubated under glasshouse condition at 26 ± 2ºC. Observations were recorded on the rapidity of disease incidence in each treatment, every alternate days and the per cent disease incidence was calculated by using the following formula given by Kranz and Rotem, (1988).

$$\% \text{ disease incidence} = \frac{\text{No of diseased plants / pot}}{\text{Total no. of plants / pot}} \times 100$$

**Histopathological studies**

The location of fungi in seed was studied by employing component plating technique (Maden *et al.*, 1975). Naturally infected soybean seed samples, of variety JS335 was used for the study. 10 seeds were washed four times with tap water then surface sterilized in one per cent sodium hypochlorite solution for one minutes. These seeds were again washed with sterile water and soaked in water for 1h and then the seeds were dissected aseptically using sterile needle and forceps. The separated seed parts *viz*., pericarp, embryo, endosperm, hilum were plated immediately before drying on potato dextrose agar plates. The plates were incubated at 20 ± 2ºC for seven days, the seed component were examined under stereoscopic binocular microscope for the presence of fungal structures in different seed parts.

## RESULTS AND DISCUSSION

**Molecular diagnosis of seed-borne fungal infection of soybean through PCR using fungal specific primer**

The total DNA was isolated by using modified CTAB method and further amplified using fungal specific primers *i.e.*, ITS1 and ITS4 in a gradient thermocycler. The results indicated that the presence of bands with a size of 500-550bp. No amplified product was observed in case of healthy seed sample (Fig.1). This was confirmed with the similar study conducted by Saddala *et al*., (2010), she has studied internal transcribed spacers region of rDNA amplification with specific ITS1 and ITS4 in case of *Sclerotium rolfsii* and produced approximately 650 to 700bp in all the isolates.

Further the amplified product sequenced from Chromous Biotech Pvt. Ltd., Bangalore revealed the sample 1 and sample 2 genome exhibited more than 90 per cent similarities with the *C. kikuchii* genome (520bp) and *R. bataticola* genome (540bp) respectively after comparing with the NCBI database through Basic Local Alignment Search Tool (BLAST) programme. Maria *et al*. (2011) had sequenced the ITS regions showed high similarity (99-100%) to the GenBank sequences of *C. kikuchii* BRCK179. Almeida *et al.,* (2006) were also sequenced the internal spacer regions (ITS1-5.8S-ITS2) from 13 isolates and clustering analysis showed high similarity (97%-100%) to the GenBank sequences of *C. kikuchii* (AY266160, AY266161, AY152577 and AF291708). Salazar *et al*. (2000), Babu *et al*. (2007) and Sharon *et al*. (2008) were evaluated the rDNA- ITS sequencing in case of *Macrophomina phaseolina*. This study brought out a light on the use of fungal specific primers for the detection and diagnosis of seed borne infection of soybean in India.

**Effect of inoculum density, transmission studies and proving pathogenicity**

Seed transmission studies helps to confirm the seed to plant transmission to prove the pathogenicity and to design the suitable management strategy.

**Pot culture studies**

The results of pot culture studies conducted to study the seed to plant transmission of *R. bataticola*, *C. kikuchii, C. truncatum and Fusarium spp*., revealed that apparently healthy and artificially inoculated seeds exhibited severe reduction in per cent germination and disease incidence was noticed after 20 DAS. The highest disease incidence was observed in treatment $T_1$, $T_2$ and $T_3$ ($10^6$, $10^5$, $10^4$propagules/10 mL) in case of all four fungi, where as lower wilt incidence was observed in $T_4$ and $T_5$ ($10^3$ propagules per 10mL and un-inoculated apparently healthy seeds). This showed that seed inoculum levels of $T_1$, $T_2$ and $T_3$ ($10^6$, $10^5$, $10^4$ propagules/10 mL) can cause maximum disease incidence. A minimum threshold inoculum level of *R. bataticola* ($4\times10^3$ propagules per 10 mL), *C. kikuchii* ($3\times10^3$ propagules per 10 mL), *C. truncatum* ($5.5\times10^3$ propagules per 10 mL) *and Fusarium spp*. ($3.75\times10^3$ propagules per 10 mL) can reduce the per cent germination to an extent of 20 per cent (Table 1- 4). These results were agreeing with the observations of Shirshikar (1995) and Chaithra (2009).

**Location of fungi in seed**

The location of seed-borne fungi was studied by inoculating seed parts *viz.,* pericarp, embryo, endosperm and hilum on PDA by component plating technique The result indicated the presence of the seed borne pathogen in the separated seed parts *viz.,* pericarp, embryo, endosperm and hilum (Table 5). *R. bataticola* was noticed on all the parts such as pericarp, embryo and endosperm except hilum. Histopathological studies conducted by Arya *et al*. (2004) revealed that in *Macrophomina* infected seeds the fungus lies as hyphae and sclerotia in all the three layers (palisade, hypodermis and aleurone) of seeds coat as inter and intra- cellular mycelium and as sclerotia in cotyledons of infected seeds. The fungus transmits from seed to seedling in a non-systemic manner.

But *Cercospora kikuchii* infection was observed on the pericarp, endosperm and hilum only, no embryo infection is noticed. Chen *et al*. (1979) observed hyphae of *C. kikuchii* found on the seed coat and the crevices of purple stained seeds grew into the cotyledons through seed coat pores during germination, hyphae were also observed invading the ovary. Similar types of results were observed by Almeida *et al*. (2006) and Chaithra (2009). Except endosperm all other parts are infected by *Fusarium spp.Colletotrichum truncatum* was noticed on both the pericarp and hilum. *Aspergillus flavus*noticed from all the parts, but *Aspergillus niger* was observed only on pericarp and embryo.

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## REFERENCES

**Almeida, A. M. R., Piuga, F. F., Marin, S. R. R., Binneck, E., Sartori, F., Costamilan, L. M., Teixeira, M. R. O. and Lopes, M. 2006** Pathogenicity, molecular characterization, and cercosporin content of Brazilian isolates of *Cercospora kikuchii*. *Fitopatologia Brasileira* **30**: 594-602.

**Anonymous 2012.** Directors Reports and Summary Tables of Experiment. *AICRP on Soybean, Directorate of Soybean Research, Indore*.

**Arya, V. K., Vihshunavat, K. and Himanshu, N. 2004.** Detection, Location and Transmission of Seed-borne Inoculum of *Macrophomina Phaseolina* in Charcoal rot in Soybean. *J. Mycol. Pl. Pathol*. **34(3):** 233-237.

**Babu, B. K., Saxena, A. K., Srivastava, A. K. and Arora, D. K. 2007.** Identification and detection of *Macrophomina phaseolina* by using species specific oligonucleotide primers and probe. *Mycologia*. **99(6):** 797-803.

**Biswas, C., Dey, P., Satpathy, S., Sarkar, S. K., Bera, A. and Mahapatra, B. S. 2012.** A simple method of DNA isolation from jute (*Corchorus olitorius*) seed suitable for PCR-based detection of the pathogen *Macrophomina phaseolina* (Tassi) Goid. *Letters in Appl. Microbiol*. **56:** 105-110.

**Chaithra, M. and Laxminarayanarao, M. S. 2009.** Studies on seed-borne fungal pathogens of chickpea and their management with special reference to *Fusarium solani* (Mart.) *M. Sc. (Agri.) Thesis, Univ. Agric. Sci.,* Dharwad, Karnataka (India).

**Chen, M. D., Lyda, S. D. and Halliwell, R. S., 1979,** Infection of soybean with conidia of *Cercospora kikuchii*. *Mycologia*. **71**: 1158-1165.

**Doyle, J. J. and Doyle, J. L. 1990.** Isolation of plant DNA from fresh tissue. *Focus*. **12**: 13-15.

**Ellis, M. L., Broders, K. D., Paul, P. A. and Dorrance, A. E. 2011.** Infection of soybean seed by *Fusarium graminearum* and effect of seed treatments on disease under controlled conditions. *Pl. Dis.*, **95(4):** 401-407.

**Erlich, H. A., Gelfand, D. H. and Saiki, R. K. 1988.** Specific DNA amplification. *Nature*. **331**: 461-462.

**Irina, S. D., Alexei, G. K., Monika, K., John, B., George, S. and Christian, P. K. 2005.** An oligonucleotide barcode for species identification in Trichoderma and Hypocrea. *Fungal Geneticsand Biology*. **42**: 813-828.

**Karuna, V. and Kolte, S. J. 2005.** Essentials of phytopathological techniques . pp. 25-26.

**Kranz, J. and Rotem, J. 1988.** Experimental Techniques in Plant Disease Epidemiology. pp 35-50.

**Maden, S., Singh, D., Mathur, S. B. and Neergaard, P. 1975.** Detection and location of seedborne inoculum of *Ascochyta rabei* and its transmission in chickpea. *Seed Sci. Tech*. **3**: 667-671.

**Maria, C. L., Maria, G. L. R., Maria C. V., Roxana, M., Anabel, S., Mónica, M. and Ana, M. G. 2011.** Genetic diversity of *Cercospora kikuchii* isolates from soybean cultured in Argentina as revealed by molecular markers and cercosporin production. *Mycopathologia,* **171(5):** 361-371.

**Molouba, F., Guimier, C. and Berthier, C. 2001.** Detection of Bean Seed-borne Pathogens by PCR. *Acta Horticulturae*. **546**: 603-607.

**Saddala, D. P., Shaik, T. B., Narreddy, P. and Gari, E. R. 2010.** Molecular variability among the isolates of *Sclerotium rolfsii* causing stem rot of groundnut by RAPD, ITS-PCR and RFLP. *Eur Asia. J. Bio. Sci*. **4**: 80-87.

**Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. and Erlich, H. A., 1988,** Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*. **239**: 487-491.

**Salazar, O., Julian, M. C. and Rubio, V. 2000,** Primers based on specific rDNA-ITS sequences for PCRdetection of *Rhizoctonia solani, R. solani* AG 2 subgroups andecological types and binucleate *Rhizoctonia*. *Mycol. Res.* **104(3):** 281-285.

**Sharon, M., Kuninaga, S., Hyakumachi, M., Naito, S. and Sneh, B., 2008,** Classification of Rhizoctonia spp. using rDNA-ITS sequence analysis supports the genetic basis of the classical anastomosis grouping *Mycoscience*. **49**: 93-114.

**Shirshikar, S. P. 1995.** Studies on seed borne nature and cultural aspects of *Colletotrichum truncatum* (Schw.) Andrus and Moore; incitant of anthracnose disease of soybean (*Glycine max* (L.) Merrill). *Ph. D. Thesis. Uni. Agric. Sci.* Banglore, Karnataka (India).

**Sinclair, J. B. and Backman, P. A. 1989.** *Compendium of Soybean Diseases*.(3rd Edition).American Phytopathological Society, St. Paul, Minnesota, USA, p. 106.

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