www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2022; 11(4): 901-905

www.thepharmajournal.com
Received: 08-02-2022
Accepted: 24-03-2022

Santosh Kumari
Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India

Dr. AK Meena
Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India

Jasveer Singh
Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India

Vikash Kumar
Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India

Corresponding Author:
Jasveer Singh
Department of Plant Pathology, College of Agriculture, SKRAU, Bikaner, Rajasthan, India

Isolation, purification and management of *Macrophomina phaseolina* through important bioagents *in vitro*

Santosh Kumari, Dr. AK Meena, Jasveer Singh and Vikash Kumar

Abstract
Background: Charcoal rot of fenugreek caused by *Macrophomina phaseolina* is most destructive disease of all fenugreek growing areas of Rajasthan, under severe infestation it cause 42.56% in kernel and 36.92% losses in pod yield. Recently, central insecticide board (cib), faridabade banned 27 pesticides including some important fungicides which are extensively used in plant disease management. Therefore, in the present investigation, our main emphasis was to find out some bioagents for management of charcoal rot of fenugreek.
Method: Two fungal bioagents *viz., Trichoderma harzianum, Trichoderma viride* and two bacterial bioagents *viz., Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated in different concentration against *Macrophomina phaseolina* in *in vitro.*
Results: Among the all bioagents, *T. harzianum* was found most effective in controlling the mycelium growth. These treatments can provide an effective and economical management of charcoal rot disease for fenugreek cultivators.

Keywords: Fenugreek, *Macrophomina phaseolina,* charcoal rot, bioagents

Introduction
Fenugreek (*Trigonella foenum-graecum* L.) also known as *Methi* belongs to family *leguminoceae*. Fenugreek believed to be originated from South East Europe and West Asia is cultivated throughout India and other parts of world for leafy vegetables, condiment, medicinal purposes and fodder also. It is rich source of minerals, protein, vitamin A and C. Fenugreek seed is also used as dye making and for extraction of alkaloids or steroids. The dried leaves and flowers are used for flavour. The leaves (per 100 gram of edible portions) contain moisture (per 100 gram of edible portions) 86.1 g, thiamine 0.05 mg, fat 0.9 g, calcium 360 mg, protein 4.4 g, oxalic acid 13 mg, fiber 1.1 g, iron 17.2 mg, potassium 51 mg, mineral 1.5 g, sulphur 167 mg, carbohydrates 6.00 g, vitamin A 6450 IU, magnesium 67 mg, nicotinic acid 0.7 mg, sodium 76.1 mg, vitamin C 54 mg, phosphorus 51 mg and chlorine 165 mg (Bose and Som, 1986) [3].
India is known as the “Land of Spices″. It is the largest producer, consumer as well as exporter of spices and spice products in the world. Rajasthan and Gujarat are known as ″Seed Spices Bowl″ and contributes more than 80 per cent of total seed spices production in India. Out of the total sixty three spices grown in India, twenty are being classified as seed spices. Among them, cumin, coriander, fennel and fenugreek are considered as major seed spices.
India stands first in the production of fenugreek with total acreage of 1.20 lakh hectares and production of 1.88 lakh tonnes with average productivity of 1566.23 kg/ha. In India more than 80 per cent area and production of is contributed by Rajasthan state alone. In Rajasthan first rank in the production of fenugreek with total acreage of 45.31 thousand hectares and production of 62.89 thousand tonnes with average productivity of 1388.00 Kg/ha (Anonymous, 2019-20) [2].
Fenugreek is cultivated in almost all districts of Rajasthan whereas Bikaner district stands first in production with total acreage of 7046 hectares and production of 7760 tonnes with productivity of 1101 Kg/ha (Anonymous, 2018-19) [1].
Fenugreek is infected by several fungal, bacterial and viral diseases. Major fungal diseases of fenugreek are Cercospora leaf spot (*Cercospora traversian*), Charcoal rot (*Macrophomina phaseolina* (Tassi) Goid.), Wilt (*Fusarium oxysporum* Schlecht.), Downy mildew (*Peronospora trigonellae* Gaum), Rhizoctonia root rot (*Thanatephorus cucumeris* Kuhn),

Powdery mildew (*Leveillula taurica* (Lev.) Arm), Root rot (*Sclerotinia trifoliorum* Sacc.) and Rust (*Uromyces trigonellae* Pass.) etc.

Macrophomina phaseolina (Tassi.) Goid. infects more than 500 plant species worldwide (Sinclair, 1982) [11] and causes charcoal rot disease in several agronomical important crop including soyabean, maize, sorghum, cotton and fenugreek.

Charcoal rot infected seeds act as an important source of primary inoculums for new areas. Plant stand affected due to pre and post emergence infection of crop. In pre emergence stage, the fungus causes seed rot and mortality of germinating seedling while in post emergence stage seedling get blightened due to soil and seed borne infection. The pathogen may infect almost all parts of plants *viz.*, root, stem, branches, petioles, leaves and pods.

The pathogen being soil-borne and its propagules distributed randomly in soil is difficult to control by fungicides. Moreover, the fungicides are effective only on the active metabolic stage of the propagules and not on wasting structure. Soil application of fungicides is an expensive and deleterious to non target microflora. Biological control has become a critical component of plant diseases management and, it is a practical in state approach in various crops (Patel and Anahosur, 2001) [8]. Bioprotectants provide unique opportunity for crop production, since they grow proliferate, colonize and protect the newly formed plant parts, to which they were not initially applied (Harman 1991) [4]. Bioagents isolated during the course of studies were tested for antagonism to *Macrophomina phaseolina*. The bioagents indicating such properties were utilized for possible biological control of fenugreek charcoal rot by addition of the cultures of inter antagonists to infected soil.

Root rot of fenugreek (*Trigonella foenum-graecum* L.), caused by *Rhizoctonia solani* has become an important constraint to the growers of fenugreek in Rajasthan. Disease incidence varied from 26.50 to 36.17 per cent in five surveyed districts of Rajasthan. To know the status of the disease in Rajasthan, survey was carried pot in five districts of Rajasthan. Maximum disease incidence was recorded in Jaipur district (36.17%) followed by Nagaur (35.55%), Jhunjhunu (33.90%), Jodhpur (32.28%) and Sikar (26.50%). In Rajasthan, the overall average of disease incidence was 32.39 per cent in 50 fields of 10 villages of five districts (Yadav *et. al.*, 2019) [14].

The disease is wide occurrence in sandy soil of Rajasthan, where the climatic conditions are dry and temperature remains high. Therefore, it was very important to undertaken the studies to find out the cause for disease development and its suitable management measures. Though the disease has been investigated in other parts of country, yet the importance of study cannot be ignored because of the climatic conditions of Rajasthan, the present investigation was undertaken to manage of this important disease through new fungicides.

Material and Methods

Collection, isolation, purification and pathogenicity of *M. phaseolina*

Collection and Isolation of *Macrophomina phaseolina*

Collection of root rot infected samples and isolation of *Macrophomina phaseolina* from infested fenugreek plants were carried out from field. The infested root samples were gently washed in tap water for removing the soil particles adhering on root surface. The washed root parts were cut into small pieces and surface sterilized in 0.1% sodium hypochloride solution in Petri plates for 1-2 minutes followed by repeated washing in sterilized distilled water. The surface sterilized pieces were transferred aseptically on Potato Dextrose Agar (PDA) medium (Appendix - I) in Petri plates and kept in BOD incubator for 7 days at 28±2°C for growth of the pathogen.

Purification and pathogenicity test

In order to obtain the pure cultures of *Macrophomina phaseolina*, single hyphal tip method was used. The hyphal suspension of the isolate was prepared in sterilized distilled water so as to obtain 5-6 hypha per microscopic field (10X). The hyphal suspension was spread over the surface of sterilized 2 per cent plain agar medium in Petri plates and incubated at 28±2°C for 18-22 hours. The single hyphal piece was observed under low power objective and cut through dummy objective. Such pieces were transferred separately on Potato Dextrose Agar (PDA) slants with the help of an inoculating needle and incubated at 28±2°C for seven days. These cultures were observed under microscope and slants stock of cultures were prepared and kept in refrigerator for further studies. Pathogenicity of the purified cultures of *M. phaseolina* was tested by growing fenugreek plants. In pots containing pathogen infested soil. For this purpose, sand maize meal medium (2:1) was prepared in 2:1 proportion, properly moistened and transferred in 250 ml Erlenmeyer flasks which were sterilized at 15 psi for 30 min. The prepared sterilized media was inoculated with respective *M. phaseolina* culture and incubated at 28±2°C for 10 days. The sand maize media inoculated with respective *M. phaseolina* were added to soil at 20 g kg^{-1} soil and mixed thoroughly. The inoculated soils were transferred to clean earthen pots properly moistened and allowed for 72 hours to stabilize the inoculum before sowing of fenugreek seeds. Ten healthy seeds of fenugreek were sown in each pot. Suitable control was maintained in which fenugreek seeds were sown in pathogen free soil. The pots were watered regularly to maintain suitable moisture regime. The charcoal rot symptoms developed in seedlings were recorded periodically. Re-isolation of the pathogen was made from infested seedlings, identified under microscopic observation and maintained for further uses.

Isolation of antagonistic microflora

All the antagonistic fungal and bacterial microbes were isolated from fenugreek rhizospheric soil. Isolated of fungal antagonistic *Trichoderma species* were cultured on Potato Dextrose Agar media, while, bacterial antagonists like *bacillus subtillis* and *pseudomonas fluorescens* maintained on Nutrient Agar (NA) and Pseudomonas Agar Fluorescens (PFA) (Hi-Media, Mumbai) media. The composition of media is mentioned in appendix - 1.

Isolation of fungal antagonists

In order to isolate fungal antagonists, Trichoderma Selective Media (TSM) and Martin's Rose Bengal Agar Media were used. Ten gram soil was added in 90 ml sterilized water in Erlenmeyer flask and shaken gently for 4-5 minutes. Serial dilutions were made from stock soil suspension upto 10^7 and 0.2 ml soil suspension of suitable dilution (depending on stage of soil sampling) was added to the surface of Trichoderma selective medium (Elad and Chet, 1983) and Martin's Rose

Bengal Agar media in Petri plates for isolation of *Trichoderma* spp. Soil suspension was spread uniformly with the help of glass spreader. The inoculated Petri plates were incubated at 28±2°C in BOD incubator for 7 days and the fungal colonies developed were sub cultured on potato dextrose agar media and observed under microscope for identification and further use. The composition of various media used is given in appendix-I.

Isolation of *Pseudomonas fluorescens*

For isolation of *P. fluorescens*, Pseudomonas Agar Fluorescens (PAF) Selective Media (Hi-Media) was used (King *et. al.*, 1954). The composition of this media is given in appendix-l, Stock soil solution was prepared by taking 10 g soil in 90 ml sterile distilled water in Erlenmeyer flask and shaken gently for 2 to 4 minutes. Serial dilutions were prepared from the stock soil suspension up to 10^6. A 0.2 ml soil suspension of suitable dilution was added on surface of the media in Petri plates and spread uniformly with the help of glass spreader. The inoculated Petri plates were incubated at 28±2°C for 48 hours and the colonies appeared were sub cultured on PAF media for identification and further use.

Isolation of *Bacillus subtilis*

As described above, stock soil solution was prepared by taking 10 g soil in 90 ml sterile nutrient broth in Erlenmeyer flask and shaken gently for 2 to 4 minutes. Then these were kept in water bath at 55°C for 3-5 minutes. After that, the flasks containing soil suspension were incubated for 48 hours at room temperature. Serial dilutions were prepared from the stock soil suspension using sterile distilled water up to 10^6. A 0.2 ml soil suspension of suitable dilution was added on surface of Nutrient Agar (NA) media (Appendix - l) in Petri plates and spread uniformly with the help of glass spreader. The inoculated Petri plates were incubated at 28±2°C for 48 hours and the colonies appeared were sub cultured on nutrient agar media for identification and further use.

Efficacy of each bioagents were tested against *Macrophomina phaseolina* in lab.

Evaluation of antagonistic potential of fungal antagonists

Dual culture technique was followed in order to ascertain the antagonistic capacity of *Trichoderma spp*. and other fungal antagonists. One mycelial disc (5 mm diameter) of each of the pathogen and antagonist was kept on the surface of potato dextrose agar medium in Petri plates at 5 cm apart. The inoculated Petri plates were incubated at 28±2 °C for 7 days. Three replications were kept for each fungal antagonist. In case of control, the Petri plates were inoculated with mycelial disc of the test pathogen only. The mycelial growth of test pathogen was measured after 4 days of inoculation.

Table 1: List of different bioagents and their abreviations

Bioagents	Abreviations
T_1; *Trichoderma viride*	Tv
T_2; *Trichoderma harzianum*	Th
T_3; *Pseudomonas fluorescens*	Pf
T_4; *Bacillus subtilis*	Bs
T_5; Control	

Efficacy of bioagents was tested by conducted by Dual Culture (Fungi) method and by Paper Disc (Bacteria) method. Efficacy of fungicides was determined by tested by Poison Food Technique.

Per cent growth inhibition was calculated by following formula as;

$$\text{Per cent Growth Inhibition (PGI)} = \frac{C - T}{C} \times 100$$

Where;

C = Mycelial growth of *M. phaseolina* in control (mm)

T = Mycelial growth of *M. phaseolina* in presence of antagonist (mm)

Results and Discussion

Collection, isolation, purification and pathogenicity of *M. phaseolina*

Collection and Isolation of *Macrophomina phaseolina*

Collection of root rot infected samples and isolation of *Macrophomina phaseolina* from infested fenugreek plants were carried out from field. The infested root samples were gently washed in tap water for removing the soil particles adhering on root surface. The washed root parts were cut into small pieces and surface sterilized in 0.1% sodium hypochloride solution in Petri plates for 1-2 minutes followed by repeated washing in sterilized distilled water. The surface sterilized pieces were transferred aseptically on Potato Dextrose Agar (PDA) medium in Petri plates and kept in BOD incubator for 7 days at 28±2 °C for growth of the pathogen.

Purification and Pathogenicity test

In order to obtain the pure culture of *Macrophomina phaseolina*, single spore technique was followed. Petri plates and slants of pathogen for full growth were incubated at 28±2 °C for 15 days. The purified culture used for the further studies.

Pathogenicity test was proved by atomizing the distilled water having mycelial bits of *Macrophomina phaseolina* on the foliage of 35 days old fenugreek plants in pot condition. Five days after inoculation, the symptoms started occur on inoculated plants. The symptoms observed on the roots of plants in pot were similar to observed in the field conditions. The reisolated culture from inoculated roots was confirmed as *Macrophomina phaseolina.*

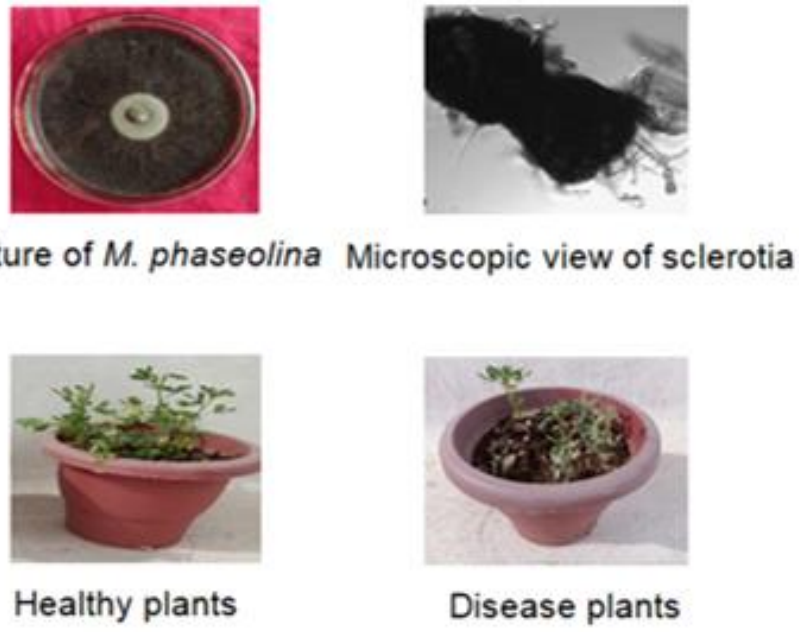

Plate 1: Pathogenicity test of *M. phaseolina* on fenugreek

The samples collected from fenugreek field showed characteristics symptoms of the disease. The nature of isolated fungus was confirmed on the basis of morphological characters particularly mycelial which had close similarly with earlier finding given by many researchers *viz.*, Sriniwas and Shankar (2017), Lakhran *et al.* (2018), Oviya *et al. (*2019) [12, 6, 7] and Reddy *et al.* (2016) [10], thus proving that the fungal pathogen was *M. phaseolina.*

Efficacy of bioagents against *M. phaseolina in vitro*

The antagonistic effect of two fungal bioagents *viz.*, *Trichoderma harzianum*, *Trichoderma viride* and two bacterial bioagents *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* were evaluated against test fungus by dual culture and paper disc method, respectively. Per cent growth inhibition was calculated based on the observation of mycelium growth of test pathogen and antagonist. The results showed in table 2, fig 1 and plate 2 observed that all the bioagents were significantly effective the inhibition of m ycelial growth of *M. phaseolina*. All the tested fungal and bacterial bioagents both fungal bioagents were significantly superior to all the bacterial bioagents. Among the tested bioagents highest growth inhibition was recorded in *T. harzianum* (74.26%) and lowest growth inhibition was observed in *B. subtilis* (38.71%).

Similar, findings was observed by Kumar and Gohel (2018) and Swamy *et al.* (2018) [5, 13] observed that two isolates of *Trichoderma spp*. four *Pseudomonas fluorescens* isolates, one *Pseudomonas putida* and one *Bacillus subtilis* were evaluated against *M. phaseolina* by Poison Food Technique and Dual Culture Method respectively. Among the bioagents tested *Trichoderma harzianum* (Th-R) was found more effective as compared to other bio-control agents and inhibited maximum fungal growth (41.86%) of *M. phaseolina*. Ramezani (2008) [9] recorded that the efficacy of four fungal bioagents *viz*., *Trichoderma hamatum, T. harzianum, T. polysporum* and *T. viride* were evaluated *in vitro* condition against the Eggplant root - rot pathogen, *Macrophomina phaseolina*. Among the bioagents, *T. harzianum* inhibit the maximum mycelium growth of 18.20 per cent compared to the minimum of 7.30 per cent by *T. hamatum*.

Table 2: Efficacy of different bioagents on mycelial growth of *M. phaseolina in vitro*

Bioagents	Mycelial growth (mm)	Growth inhibition (%)
T_1; *Trichoderma harzianum*	23.16 (34.36)*	74.26
T_2; *Trichoderma viride*	31.89 (28.74)	64.56
T_3; *Pseudomonas fluorescens*	44.50 (41.82)	50.55
T_4; *Bacillus subtilis*	55.16 (47.94)	38.71
T_5; Control	90 (71.53)	_
S.Em± CD (P = 0.05)	0.65 2.08	

*Figures in parentheses are angular transformed values

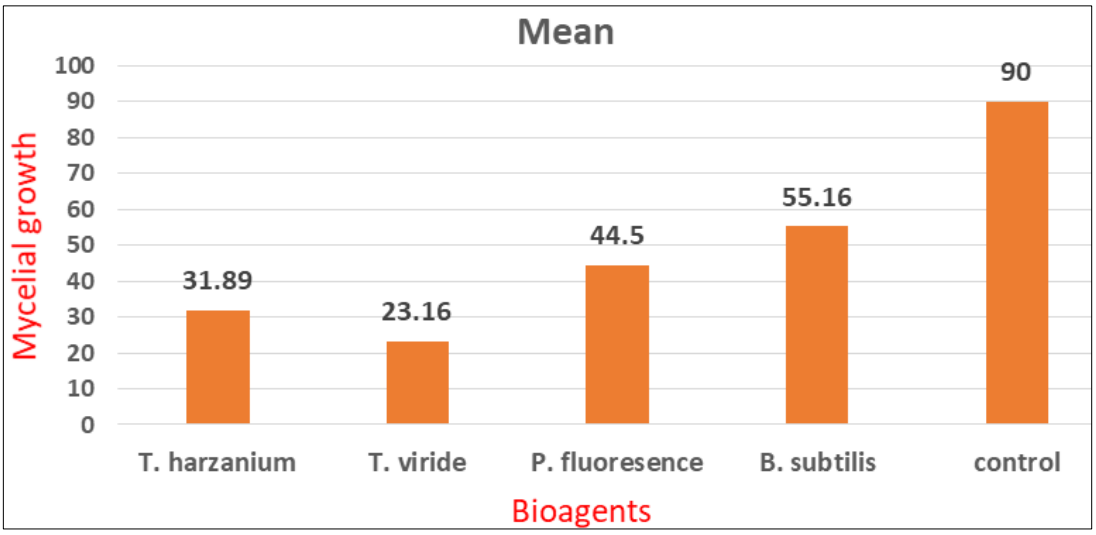

Fig 1: Efficacy of different bioagents on mycelial growth of *M. phaseolina in vitro*

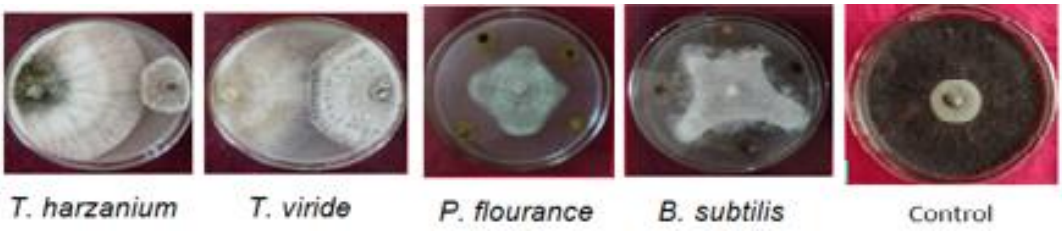

Plate 2: Efficacy of different bioagents on mycelial growth of *M. phaseolina in vitro*

Conclusion

It can be concluded unequivocally considering the results that the two fungal bioagents *T. harzianum*, *T. viride* and two bacterial bioagents *P. fluorescens*, *B. subtilis* were effectively inhibited the mycelial growth of *Macrophomina phaseolina.* Among the all bioagents, *T. harzianum* was found most effective in controlling the mycelium growth.

Acknowledgement

The authors are very much thankful to the Department of Plant Pathology, College of Agriculture, Swami Keshwanand Rajasthan Agricultural University, Bikaner for providing such a good facilities and well maintained lab.

References

1. Anonymous. Agriculture statistics, Directorate of Agriculture, Government of Rajasthan, Jaipur, 2018-19.
2. Anonymous. Directorate of Arecanut and Spices Development (DASD) Kozhikode, Kerala, 2019-20.
3. Bose TK, Som MG. Vegetable Crops in India. Naya Prakash, Kolkata (W.B.), 1986,959p.
4. Harman GE. Seed treatment for biological control of plant diseases. Crop Protec. 1991;10:166-176.
5. Kumar, Gohel. Evaluation of antagonistic potential of biocontrol agents against *Macrophomina phaseolina* (Tassi.) Goid. Causing stem and root rot of sesame [*Sesamum indicum* L.] under *in vitro and in vivo.* Int. J. Curr. Microbiol. App. Sci. 2018;7(12):1748-1754.
6. Lakhran L, Ahir RR, Choudhary M, Choudhary S. Isolation, purification, identification and pathogenicity of *Macrophomina phaseolina* (Tassi) Goid. caused dry root rot of chickpea. J. Pharma. Phytopathol. 2018;7(3):3314-3317.
7. Oviya R, Akila R, Sabarinathan KG, Mehetre ST, Rammamoorthy V. Studies on various factors influencing Growth and pathogenicity of *Macrophomina phaseolina* in blackgram. J Pharma. Phytochemistry. 2019;8(3):3545-3549.
8. Patel ST, Anahosur KH. Potential antagonism of *T. harzianum* against *Fusarium* spp. *Macrophomina phaseolina* and *Sclerotium.* J Mycol. Pl. Pathol. 2001;31(3):365.
9. Ramezani H. Biological control of root rot of eggplant caused by *Macrophomina phaseolina*. American Eurasain. J Agri. Environ. Sci. 2008;4(2):218-220.
10. Reddy AT, Gowda G, Saifulla M. Identification of sources resistant to dry root rot caused by *Macrophomina phaseolina* (Tassi.) in Chickpea (*Cicer arietinum* L.). Mysore J. Agri. Sci. 2016;50(2):369-371.
11. Sinclair JB. Compendium of soybean diseases. 2nd ed. American Phytopathol. Soc. St. Paul, MN, 1982,104p.
12. Srinivas, Shankar. Studies On pathogenicity of *Macrophomina phaseolina* on sorghum cultivars. Int. J Engi. Sci. Inv. 2017;6(12):5-8.
13. Swamy C, Naik MK, Amaresh YS, Jayalakshmi SK. Evaluation of fungicides and bioagents under *in vitro* condition Against *Macrophomina phaseolina* causing stem canker of Pigeonpea. Int. J Curr. Microbiol. App. Sci. 2018;7(1):811-819.
14. Yadav SL, Ghasolia RP, Yadav R, Yadav P. Studies on survey and epidemiology of *Rhizoctonia solani* causing root rot of fenugreek. Int. J. Seed Spices. 2019;9(2):91-95.