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Efficacy of Bio-agents and Phyto-Extracts against Seed Borne Mycoflora of Soybean (*Glycine max* L.)

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Abstract

The present study was aimed to detect and identify seed borne fungi associated with soybean seeds and subsequently determining their effect on seed germination and seedling growth. Seed mycoflora associated with soybean were detected by using agar plate and standard blotter paper method. A total of six fungal species comprising three genera i.e., *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium pallidoroseum* and *Macrophomina* sp. were isolated from soybean seed. Among these, *Fusarium pallidoroseum* and *Aspergillus flavus* were found to be the most prevalent causing considerable reduction in seed germination and seedling growth as compared to control. The effect of different bio-agents and phyto-extracts on seed mycoflora, seed germination and vigour index were evaluated. *Trichoderma viride* and Dhatura leaf extract were found to enhance seed germination, shoot length, root length and vigour index as compared to control.

Keywords: Bio-agents, phyto-extracts, seed mycoflora, seedling vigour

1. Introduction

Soybean is native of China (Shovan et al., 2008) and is grown as a commercial crop in over thirty-five countries as the major oilseed (Smith and Huyser, 1987). Among the major soybean growing countries, India ranks fourth in terms of area and fifth in terms of the production as per USDA estimates. In India, total area, production and productivity of soybean was 10.56 million ha, 11.39 million tones and 1078.6 kg ha^{-1}, respectively in 2017-18 (Anonymous, 2018). In Gujarat, soybean was cultivated on an area 1.34 million ha with the production of 1.24 million tones with productivity of 925 kg ha^{-1} (SOPA databank, 2018). Soybean is able to leave residual nitrogen effect for succeeding crop equivalent to 35-40 kg N ha^{-1}. It can tolerate mild drought as well as floods and survives better.

Diseases have been reported as a limiting factor to the production of soybean worldwide. Soybean is susceptible to a number of seed and seedling disease pathogens, most of them are seed-borne in nature and play an important role in affecting the seed quality and quantity, of which mycoflora are the largest group. Soybean seeds are known to harbour several species of seed borne fungi *viz*., *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Colletotrichum dematium*, *Chaetomium globosum*, Cercospora *kikuchi*, *Curvularia lunata*, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Penicillium* sp. and *Rhizopus stolonifer* (Shovan et al., 2008). These fungi may decrease seed germinability, cause seed discoloration, produce toxins that may be injurious to man and domestic animals and may reduce seed weight also (Neergaard, 1986).

Seed borne pathogens have been involved in seed rots during germination and seedling mortality leading to poor crop stand reduction in plant growth and productivity of crops (Kubiak and Korbas, 1999; Dawson and Bateman, 2001; Akranuchat et al., 2007). Infected seeds play considerable role in the establishment of economically important plant diseases in the field resulting in heavy reduction of crop yields. Apart from this, infected seeds act as a vehicle in carrying pathogens to uninfected areas within a country and from one country to the other (Waller, 2002).

The detection and identification of microorganisms associated with seeds of the major crops in a country is the first and major step towards efficient seed health testing system. Although, some investigations have been made intermingle on soybean in various aspects but, the information on the seed mycoflora and its effect on seed germination and seedling vigour is meager. Thus, the present study was conducted to detect and identify the seed borne fungi and to evaluate the efficiency of the different bio-agents and phyto-extracts for their relative efficacy against seed mycoflora, seed germination and seedling vigour of soybean.

2. Materials and Methods

2.1. Experimental location

The present investigation was conducted at the Department of Plant Pathology, N. M. College of Agriculture, Navsari

Agricultural University, Navsari 396 450 during 2018–2019.

2.2. Sources of experimental material

Seed samples of different prevalent varieties of soybean *viz;* NRC 37, KDS 344, JS 335 and some local varieties were collected from Krishi Vigyan Kendra, Dediyapada, NAU, Narmada, farmers field and from various markets of Navsari, Surat and Valsad districts of Gujarat. A composite sample of all these varieties was prepared by mixing the individual and and stored at room temperature at Plant Pathology Laboratory for subsequent studies. They were used for isolation of mycoflora by using Agar plate and standard blotter paper method.

2.3. Detection and identification of fungi

Surface sterilized and un-sterilized seed samples were analyzed for the detection of seed borne fungi by agar plate and blotter method following International Rules for Seed Health Testing (ISTA) with slight modifications.

2.3.1 Agar plating method

Soybean seeds were surface sterilized in 1% aqueous solution of sodium hypochlorite (NaOCl) for four minutes followed by rinsing in three change of sterile distilled water and then dried between two layers of soft paper. Ten seeds were equidistantly placed in a Petri dish containing 20 ml potato dextrose agar (PDA) media, and then incubated for seven days at 25°C. Fungal pathogen associated with soybean seed were identified following sporulation.

2.3.2. Standard blotter paper method

In this method, three pieces of filter paper were soaked in sterilized distilled water and placed at the bottom of 9 cm diameter Petri dish. Soybean seeds from composite sample were taken randomly and surface sterilized in 1% aqueous solution of sodium hypochlorite (NaOCl) for four minutes followed by rinsing in three change of sterile distilled water and then dried between two layers of soft paper, then equidistantly placed on the moist filter paper at the rate of 10 seeds per Petri dish. The Petri dishes were then incubated at 25ºC for seven days under 12 hour alternating cycle of light and darkness. After incubation, the seeds were examined under microscope for recording the seed borne fungal infections grown on the incubated seeds.

2.4. Identification of fungi

Pure cultures of individual fungal isolates were critically examined and identified. Fungi were identified based on gross colony morphology and microscopic characters. Colony identification was based on colony characteristics such as color and texture of mycelia and type of pigmentation. Microscopic characteristics of spores such as shape and colour also used to identify the pathogens associated with the soybean seed.

2.5. Effect of seed infecting fungi on seed health status

Effect of seed infecting fungi on seed health status was studied in respect of seed germinability and seedling vigour from artificially inoculated seeds with fungi isolated from naturally infected soybean seeds. Healthy seeds of soybean var. NRC 37 were artificially inoculated with each of six fungal species separately. For artificially inoculation, seeds moistened by sterilized distilled water were mixed thoroughly with 10 days old respective fungal culture growth were obtained at 25±2°C on PDA plates. One sheet of germination paper was wetted by sterilized distilled water. One hundred seeds of respective treatment were placed on first sheet evenly. Second sheet of germination paper was placed on first sheet followed by wetting it carefully. Both sheets were rolled along with wax coated paper. The rolled papers were incubated in seed germinator at 25°C for 7 days. At the end of incubation period, rolled towel papers were carefully opened. Germinated and un-germinated seeds were counted from each of the treatments. Emergence of seedling from the seeds was considered as successful germination. After end of incubation period, these seeds were used for study of seed germination and seedling vigour index. Uninoculated seeds served as control treatment for comparison.

2.6. Seed treatments with bio-agents

The effect of bio-agents on seed germination and seedling vigour index of soybean was studied by towel paper method. The experiment was laid out in CRD with four replications. To investigate the effect of different bio-agents *viz; Trichoderma viride* (0.4%), *Trichoderma harzianum* (0.4%), *Trichoderma virens* (0.4%), *Pseudomonas fluorescens* (0.5%), and *Bacillus subtilis* (0.5%), healthy seeds of soybean were first soaked in mixed spore suspension of isolated seed infecting fungi for 24 hrs and then treated by spore suspension (10^8 cfu ml^{-1}) of each of the bio-agents. Seeds treated with sterilized distilled water served as control. Total 400 seeds / treatment were plated tested. After incubation period, observations were recorded as no. of seed germinated, root length and shoot length. Seedling vigour index was calculated with the help of formula given by Mallesh et al. (2008):

Seedling vigour index (SVI)= [Mean root length (cm) + mean shoot length (cm)] X percentage germination (%)

2.7. Seed treatments with phyto-extracts

Preparation of aqueous plant extracts for the study, fresh leaves were collected, detached and surface sterilized with 1% mercuric chloride and washed first in tap water then in distilled water and blotter dried. 100 g of fresh sample was chopped and then crushed in a surface sterilized mortar and pestle by adding 100 ml distilled water (1:1 w/v). The extract was filtered through two layers of muslin cloth and was used as stock solution in the experiment. To study the effect of different phyto-extracts *viz;* Ardusi leaf extract @ 10%, Dhatura leaf extract @ 10%, Garlic leaf extract @ 10%, Neem leaf extract @ 10% and Tulsi leaf extract @ 10%, heathly seeds of soybean were first soaked in mixed spore suspension of isolated seed infecting fungi for 24 hrs and then treated by different phyto-extracts with recommended dose, separately. Seeds treated with sterilized distilled water

served as control. Total 400 seeds / treatment were plated tested. After incubation period, observations were recorded as number of seed germinated, root length and shoot length and seedling vigour index.

2.8. Statistical analysis

The data, collected under study were subjected to the statistical analysis for proper interpretation. The standard method of analysis of variance technique appropriate to the Completely Randomized Design as described by (Panse and Sukhatme, 1967) was used. The data were analyzed with the technical help received from Department of Agricultural statistics, NMCA, NAU, Navsari. The treatment differences were tested by employing 'F' test at five per cent level of significance on the basis of null hypothesis. The appropriate standard error of mean (SEm±) was calculated in each case and the Critical Difference (CD) at five per cent level of probability were worked out to compare the two treatment means, where the treatment effects were found significant under 'F' test. The co-efficient of variation percentage (CV %) was also worked out for all the cases.

3. Results and Discussion

3.1. Prevalence of seed borne fungi on soybean

The seed borne pathogens *viz*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium pallidoroseum* and *Macrophomina* sp. were found predominant on seeds of soybean in agar plate and standard blotter paper method (Table 1). Rao et al. (2015) reported that similar fungi are associated with seeds of soybean. Similarly, Ramesh et al. (2013) found that *Macrophomina phaseolina, Fusarium oxysporum, Aspergillus niger, Aspergillus flavus, Phoma* spp and Sclerotinia sclerotiorum were associated with seeds.

3.2. Effect of seed borne fungi on seed health status

Assessment by artificially inoculation of soybean seeds separately by six different fungi revealed significant adverse effect on seed germination, shoot and root length, and thereby seedling vigour index (Table 2). Seed inoculated by *Fusarium pallidoroseum* caused lower per cent germination (49.00%) followed by *Aspergillus flavus* (55.00%), *A. versicolor* (59.00%), *Fusarium oxysporum* (61.00%), *A. niger* (65.00%) and *Macrophomina* sp. (68.00%). Overall, fungi induced 31.57 to 48.42, 48.20 to 71.19 and 44.97 to 56.83% reduction in seed germination, shoot length and root length over healthy seeds, respectively. The vigour index was reduced in all the inoculated seeds, by each fungus from 456.68 to 805.26 in comparison to control (2162.80). The present results are similar as reported by Singh and Thapliyal (1999) who found that all the test fungi caused significant inhibition in germination and production of diseased seedlings in soybean cv PK-262. Seed germination and seedling growth greatly influenced by seed mycoflora reported by Christensen and Kaufmann, 1965; Rati

Table 1: Mycoflora associated with naturally infected soybean seeds

Isolate No.	Fungi	Agar plating method		Standard blotter paper method	
		SS	US	SS	US
1.	*Aspergillus flavus*	*	*	*	*
2.	*Aspergillus niger*	*	*	*	*
3.	*Aspergillus versicolor*	*	*	-	*
4.	*Fusarium oxysporium*	*	*	*	*
5.	*Fusarium palli-doroseum*	*	*	-	*
6.	*Macrophomina* sp.	*	*	-	*

SS: Surface sterilized; US: Un-sterilized; *: Present; -: Absent

Table 2: Effect of seed borne fungi on seed health status

Fungi	Seed germination (%)	Decrease in seed germination over healthy seed (%)	Shoot length (cm)	Decrease in shoot length over healthy seed (%)	Root length (cm)	Decrease in root length over healthy seed (%)	Seedling vigour index (SVI)*
Aspergillus flavus	55.00	42.10	4.08	69.55	4.21	55.02	456. 68
Aspergillus niger	65.00	31.57	4.97	62.91	4.31	53.95	598. 20
Aspergillus versi-color	59.00	37.89	3.86	71.19	5.15	44.97	531. 45
Fusarium ox-ysporum	61.00	35.78	6.80	49.25	4.67	50.10	700. 41
Fusarium palli-doroseum	49.00	48.42	5.75	57.08	4.04	56.83	479. 31
Macrophomina sp.	68.00	28.42	6.94	48.20	4.89	47.75	805. 260
Control	95.00	-	13.40	-	9.36	-	2162.80
SEm±	1.41		0.13		0.03		20.61
CD (*p*=0.05)	4.18		0.38		0.20		61.04
CV %	4.38		3.96		2.64		5.03

* Values are means of four replications

and Ramalingam, 1974; Prasad, 1980. These results are more or less in agreement with Lee (1984) and Tripathi and Singh (1991). Lee (1984) reported that germination was reduced in soybean due to *Fusarium oxysporum.* Tripathi and Singh (1991) reported that *Aspergillus niger* was the most damaging to germination of soybean seed and elongation of seedling roots. Similarly, Raj et al. (2002) also identified the species of *Aspergillus*, *Alternaria*, *Rhizoctonia*, *Fusarium*, *Phoma* and *Chaetomium* were affecting germination and emergence of soybean seeds.

3.3. Management of seed borne fungi by means of bio -agents

Seed treatment with bio agents were found effective in reducing the effect of seed mycoflora and increase the seed germination, shoot length, root length and seedling vigour index over the control. Data presented in the Table 3 revealed overall bio-agents recorded 35.84 to 64.15, 16.80 to 77.86, and 16.50 to 84.62% increase in seed germination, shoot length and root length, respectively over control. Seed treatment with *Trichoderma viride* recorded maximum seed germination (87.00%) followed by *Trichoderma harzianum* (83.00%), while in *Trichoderma virens, Pseudomonas fluorescence* and *Bacillus subtilis* recorded 80.00, 76.00 and 72.00% seed germination, respectively. The results in terms of shoot and root length with seedling vigour index, all the treatments showed larger shoot length, root length and seedling vigour index as compared to control. Seed treatment with *T. viride* recorded maximum shoot length (11.01 cm), root length (9.73 cm) and seedling vigour index (1807.18). Similarly, T. *harzianum* (10.64 cm, 9.14 cm and 1643.27), *T. virens* (10.14 cm, 8.63 cm and 1502.15), *P. fluorescens* (7.23 cm, 6.14 cm and 1016.82) and *B. subtilis* (7.34 cm, 8.48 cm and 1139.55) also recorded more shoot length, root length and seedling vigour index, respectively as compared to control (6.19 cm, 5.27 cm and 607.88). Uses of antagonistic microbes to control the fungal pathogens were reported by various workers (Jos et al*.,* 1995; Omar and Abd Alla*,* 1998; Sharma et al., 1995). Indira et al. (2006) found 86.6% seed germination and 2478.47 seedling vigour index in seed treatment with *Trichoderma viride* in sorghum. Similarly, Rajeswari and Kumari (2009) observed that seed treatment with *Trichoderma viride* @ 6 g kg^{-1} gave significant increase in germination 91.00% and seedling vigour index (2601) in soybean.

Table 3: Management of seed borne fungi by means of bio-agents

Treatment	Conc.	Seed germination (%)	Increase in seed germination over healthy seed (%)	Shoot length (cm)	Increase in shoot length over healthy seed (%)	Root length (cm)	Increase in root length over healthy seed (%)	Seedling vigour index (SVI)*
Trichoderma viride	0.4%	87.00	64.15	11.01	77.86	9.73	84.62	1807.18
Trichoderma harzianum	0.4%	83.00	56.60	10.64	71.89	9.14	73.43	1643.27
Trichoderma virens	0.4%	80.00	50.94	10.14	63.81	8.63	63.75	1502.15
Pseudomonas fluorescens	0.5%	76.00	43.39	7.23	16.80	6.14	16.50	1016.82
Bacillus subtilis	0.5%	72.00	35.84	7.34	18.57	8.48	60.91	1139.55
Control	---	53.00	---	6.19	---	5.27	---	607. 88
SEm±		1.35		0.08		0.05		26.70
CD (*p*=0.05)		4.05		0.26		0.17		79.96
CV %		3.60		1.98		1.49		4.15

*: Values are means of four replications

3.4. Management of seed borne fungi by means of phyto-extracts

Seed treatment with phyto-extracts also recorded 37.25 to 56.86, 04.45 to 45.76 and 06.59 to 47.91% increase in seed germination, shoot length and root length, respectively over control (Table 4). Seed treated with Dhatura leaf extract recorded maximum seed germination (80.00%) which was at par with Neem leaf extract (79.00%) and Garlic leaf extract (77.00%). Whereas, Tulsi and Ardusi leaf extracts recorded 73.00, and 70.00% seed germination, respectively. The results in terms of shoot and root length with seedling vigour index, all the treatments showed larger shoot length, root length and seedling vigour index as compared to control. Seed treated with Dhatura leaf extract recorded maximum shoot length (9.81 cm), root length (8.52 cm) and seedling vigour index (1467.06). Similarly, Neem leaf extract (9.29 cm, 7.89 cm and 1357.47), Garlic leaf extract (7.03 cm, 6.14 cm and 1014.11), Tulsi leaf extract (8.33 cm, 7.38 cm and 1147.67), and Ardusi leaf extract (8.16 cm, 7.16 cm and 1072.37) also recorded more shoot length, root length and seedling vigour index, respectively as compared to control (6.73 cm, 5.76 cm and 637.01). Several botanicals have been reported to increase seed germination and vigour index by reducing the pre and post emergence mortality in several crops including soybean (Prasad and Simlot, 1982; Sivan and Chet, 1986; Farzana and Ghaffar, 1991; Haque and Ghaffar, 1992; Jacob

Table 4: Management of seed borne fungi by means of phyto extracts

Treatment	Conc.	Seed germination (%)	Increase in seed germination over healthy seed (%)	Shoot length (cm)	Increase in shoot length over healthy seed (%)	Root length (cm)	Increase in root length over healthy seed (%)	Seedling vigour index (SVI)*
Ardusi leaf ex-tract	10%	70.00	37.25	8.16	21.24	7.16	24.30	1072.37
Dhatura leaf extract	10%	80.00	56.86	9.81	45.76	8.52	47.91	1467.06
Garlic leaf ex-tract	10%	77.00	50.98	7.03	04.45	6.14	06.59	1014.11
Neem leaf ex-tract	10%	79.00	54.90	9.29	38.03	7.89	36.97	1357.47
Tulsi leaf extract	10%	73.00	43.13	8.33	23.77	7.38	28.12	1147.67
Control	---	51.00		6.73		5.76		637. 01
SEm±		1.49		0.09		0.05		21.14
CD (*p*=0.05)		4.46		0.28		0.15		63.31
CV %		4.16		2.29		1.47		3.79

* Values are means of four replications

and Sivaprakasam, 1994; Kaur and Mukhopadhyay, 1992). Sharma et al. (2013) also reported the antifungal effect of extract obtained from *Datura stramonium* against *Aspergillus flavus*, *Aspergillus niger*, *Fusarium culmorum* and *Rhizopus stolonifer*. Similar finding was also observed by Uma (2009) who found that leaf extract of *D. stramonium* was effective against *Aspergillus niger* and *Fusarium* sp. Shivpuri et al. (1997) reported that extract of *Datura stramonium* showed antifungal properties against *F. oxysporum* fungus. Similarly, Ghosh et al. (2020) observed that seed treated with neem and tulsi leaf extract gave 80.00% and 76.00% seed germination in mustard.

4. Conclusion

In recent years much attention has been given to non-chemical systems for seed treatment as well as to achieve the protection against seed borne pathogens. The present study has shown that major fungi found associated with seeds of soybean were *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus versicolor*, *Fusarium oxysporum*, *Fusarium pallidoroseum* and *Macrophomina* sp. and these were adversely affecting the seed germination, shoot and root length as well as seedling vigour index of soybean. Among bio-agents and phyto-extracts tested, the treatment of *Trichoderma viride* @ 0.4% and Dhatura leaf extract @ 10% were effective in reducing seed borne fungi, increasing seed germination and seedling vigour index.

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