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Identification of groundnut genotypes and wild species resistant to stem rot using an efficient field screening technique

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Abstract:
Stem rot of groundnut caused by *Sclerotium rolfsii* Sacc. is one of the major constraint to groundnut production in many countries. The fungus is ubiquitous, soil inhabitant and non-target. Screening of groundnut genotypes for resistance to stem rot under field conditions is complicated by the non-uniform spatial distribution of the pathogen. While development and maintenance of artificial sick plot with optimum inoculums load under field conditions are also difficult because of sensitivity of the pathogen to temperature, humidity, soil type, cropping system and host preference. In the present study, a screening technique under field conditions with high disease pressure has been reported. Twenty-five wild *Arachis* accessions and 178 F_3 progenies along with two parents were screened under pot and field conditions, respectively for resistance to stem rot. Two wild *Arachis* accessions and three F_3 lines were found resistant to stem rot with mortality less than 20% and 10%, respectively.
Key words: Groundnut, *Sclerotium rolfsii*, Stem rot, wild species, post-rainy

Introduction

Groundnut (*Arachis hypogaea* L.) is an important oilseed and food crop, cultivated on 25.44 m ha globally with a total production of 45.22 m tons during 2013 (FAOSTAT, 2014). Stem rot of groundnut caused by *Sclerotium rolfsii* Sacc. is one of the major constraint to groundnut production in many countries in warm and humid areas (Bagwan, 2011). Stem rot is also known as southern-blight, southern-stem rot, *Sclerotium* rot, or white mold and is widely distributed in India and USA. Besides, it causes serious losses in Bolivia, China, Egypt, Taiwan, and Thailand. *S. rolfsii* Sacc. (telomorph: *Athelia rolfsii* Tu and Kimbrough) is a neurotropic soil-borne fungal pathogen that infects about 500 plant species including groundnut (Aycock, 1966, Punja, 1988). Stem and pod rots caused by *S. rolfsii* cause economic losses in soybean, groundnut, sugar beet, pepper, tomato and potato. In groundnut, up to 30 percent yield losses were recorded in farmers field (Anonymous, 2012), but can reach over 80% in heavily infested fields (Porter *et al.*, 1982, Mehan and Macdonald, 1990). *S. rolfsii* also causes indirect losses such as reduction in both dry weight and oil content of groundnut kernels besides downgrading the quality of pod and fodder. Stem rot is a persistent soil borne disease throughout India and its incidence is increasing gradually even at maturity stage of the groundnut crop. Though *S. rolfsii* resides both on seed and soil, soil-borne nature of the disease is more prevalent than seed borne (Kumar *et al*., 2013). The occurrence of the disease is more visible at 30 to 45 days after germination and at the time of harvest under rain-fed situations due to low and erratic distribution of rainfall. The fungus is ubiquitous, soil inhabitant, non-target and one of the most destructive plant pathogen. It preferentially attacks stem, but it can infect any part of the plant including root, leaf, flower and fruit. On erect plant, yellowing and wilting are usually preceded by light to dark brown lesions at collar region of the stem adjacent to the ground. Drying or shriveling of the foliage and ultimately death of the plants occur after wilting. Characteristic sclerotia, at first white and later brown to black, are produced on mats of mycelium on stem surface of the plant adjacent to soil or on soil surface. *S. rolfsii* penetrates non-wounded host seedlings directly by the formation of appressoria. Penetration may also be effected through natural openings such as lenticels and stomata. The fungus is both inter and intra cellular. Batmen and Beer (1965) reported that both Oxalic acid and Pectic enzymes are involved in the destruction of host tissues by the fungus and that two fungal products acting together are more effective than either alone.
Chemical and cultural practices have been the predominant means for the management of this disease (Porter *et al.,* 1982). Persistence of the pathogen in soil and wide host range (about 500 species) often limits the effectiveness of chemical and cultural control of stem rot disease (Shew *et al.,* 1987). However, such cultural practices coupled with resistant cultivars can increase the efficiency of the disease management (Shew *et al.,* 1984). Host plant resistance is an important component of such an approach which is currently not fully exploited in groundnut. Screening of

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segregating populations in large scale for resistance under field conditions with natural infestation is complicated by the non-uniform spatial distribution of the pathogen (Shew*et al.,* 1984). As a result, consistent and reliable data is difficult to obtain in field conditions under natural infestation. While development and maintenance of artificial sick plot with optimum inoculums load under field conditions for screening of genotypes and breeding lines in large scale are also difficult because of sensitivity of the pathogen to temperature, humidity, soil type, cropping system and host preference. Thus, limiting the success of breeding groundnut variety with resistance to stem rot. Certain genotypes (e.g., ICG 12083) though have shown resistance in the field, but are less resistant in greenhouse tests (Singh *et al*., 1997). Promising genotypes and segregating progenies should be evaluated in field, microplot and greenhouse environments to identify true resistant lines and characterize components of resistance (Shew *et al.*, 1987). In our studies, an attempt was made to develop a simple, repeatable screening technique for resistance to stem rot under field conditions and identify resistant wild *Arachis* species and breeding lines.

Materials and Methods:

Multiplication of *S. rolfsii*:

Directorate of Groundnut Research (DGR), Junargadh is maintaining isolates of *S. rolfsii* collected from groundnut growing areas of Gujarat and Maharashtra state of India. ICAR-DGR has characterized all these isolates (Bagwan, 2011).Among these most virulent isolate was collected from the Plant protection Unit and subsequently maintained by the author. The culture was sub-cultured in 90 mm Petri dishes containing standard potato dextrose agar (PDA) medium and showed rapid growth of the fungus (Fig.1). The fungus was further mass multiplied on sorghum grains, where it multiplied faster than other substrates tested (Vinod Kumar *et al.*,2012). Sorghum grains (about 500 g) were boiled in tap water for 30 minutes and autoclaved for 15 minutes at 15 lb pressure. Sterile sorghum grains were inoculated with mycelium of *S. rolfsii* taken from margin of actively growing cultures in PDA medium using borer of 10 mm in diameter. The inoculated poly bags were incubated for 8 to 10 days at room temperature for healthy growth of the fungus and for further use (Fig.2). The fungus multiplied on sorghum grain was released to the crop at specified crop growth stage.

Standardization of screening technique for stem rot resistance

A. Field conditions

A total of 178 F_3 progenies derived from crossing GG 20 and CS 19 along with two parents were screened for resistance to stem rot under natural field conditions at DGR, Junagadh during *Rabi* / summer (January to May), 2015. DGR is situated between 21.52^{o}N latitude and 70.47^{o}E longitude at an elevation of 107 meters above mean sea level with an average rainfall of 1520.3 mm. The monthly mean maximum and minimum temperature ranged from 43.2^{o}C (May) to 5.5^{o}C (January) and mean relative humidity varies from 88.0 percent (July) to 35.0 percent (march) (www.jau.in). Genotypes were sown in single line on 2 meter-width bed with a spacing of 30 cm between lines and 10 cm between plants (Fig.3). Observation on initial plant population of each genotype was recorded on 35 days after sowing. Later, each block of 10 lines (3 m length x 2 m width) was mulched with sun-dried, groundnut fodder, collected from field after harvesting of crop in previous seasons (Vinod Kumar *et al.*, 2012). After mulching with groundnut fodder (Fig.4) uniformly (approximately one inch in height throughout the block), blocks were watered to field capacity at 24 hours interval for consecutive five days to ensure maximum soil moisture and softening of groundnut fodder. Care was taken to ensure uniform distribution of groundnut fodder in each block, fodder was not floated into one place in the block during watering and moist to its maximum capacity. Blocks were watered sufficiently immediate before inoculation. *S. rolfsii* inoculum grown on sorghum grain were mixed with sterilized sand (1:1 ratio) just before inoculation to ensure easy and uniform delivery of inoculum. Each block was inoculated with *S. rolfsii* multiplied on one kg of sorghum grain to ensure sufficient and equal inoculum load to each block as well as plant (Fig. 5). Inoculation was done in the evening hours to avoid exposure of inoculum to the sun and loss of moisture from sorghum grain. After inoculation a rectangular structure (3.0m x 1.82 m width x 0.8 m height) fabricated with 0.75 mm diameter round galvanized pipe was placed on each block (Fig.6). The very basic purpose of the fabricated rectangular structure is to support polythene sheet and green shade net above the certain height from plants which creates a low-cost mini poly-house structure. Further, each rectangular structure was covered with 1 mm thick low density polythene sheet and all sides of polythene were covered with soil to avoid loss of moisture from block (Fig.7). Subsequently each block was further covered with 5.2 mm green shade net (providing 75% shade) over the polythene sheet to avoid excess heating inside the structure (Fig.8). Maximum care was taken to ensure temperature $\leq$ 37 ^{o}C and relative humidity more than $\geq$ 90%

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inside the polythene covered structure for maximum growth and prolonged mycelial stage of fungus to maximum period (96 – 120 hours after inoculation). Optimum temperature was maintained inside the rectangular structure covered with polythene sheet by raising of polythene sheet in two corners of opposite directions according to the direction of wind during hot sun shine hours (Fig.9). Blocks were watered to its maximum capacity prior to closing of polythene sheet in corners or otherwise in the evening hours every day. Simulated conditions were maintained for continuous five days and after that crop was exposed to the normal conditions. Mortality of plants was recorded 15 days after inoculation and expressed in percentage.

B. Pot conditions

Wild *Arachis* species, native to South America are very difficult to establish and multiply under natural field conditions. Hence, care was taken to raise these wild *Arachis* species in a content facilities with special kind of pots. Wild *Arachis* species were sown in concrete pot or cement ring (61 cm diameter and 46 cm height with sealed bottom and two inch thickness of ring) (Fig.10). Such type of cement pots are easily available in local market, cost effective and can be used repeatedly over years. Each pot was filled up to 30 cm height with mixture of soil and sand in 1:1 ratio. Seeds of 25 accessions of 10 wild *Arachis* species, maintained at Genetics and Cytogenetics Section, DGR were used in the screening. For breaking seed dormancy, seeds of wild species were soaked overnight in Ethrel (2-chlorophosphonic acid) (0.5 ml / liter of tap water) and sown in the next day morning. Thirty seeds of each accessions were sown in three pots, having10 seeds in each pot, comprising three replications. Mulching of groundnut fodder and inoculation technique were same as described for screening at field conditions. Each pot was inoculated with *S. rolfsii* multiplied on 100 g of sorghum grain (Fig.11). Cement pot was covered with polythene sheet as well as green shade net immediately after inoculation and tied with rope to avoid damage / flying away in wind (Fig.12). Optimum temperature was maintained in pots by opening polythene sheet and green-shade net in one side during peak sun shine hours and again covered fully after watering in the evening. Simulated conditions were maintained for continuous five days and after that crop was maintained under normal field conditions. Mortality of plants was recorded 15 days after inoculation and expressed in percentage.

Results and Discussion

Selection of optimum conditions for field screening of stem rot:

Time and place of screening for resistance to stem rot is very crucial for generation of reliable data under field conditions as temperature, humidity and soil type as well as conditions of a particular place used to play major role for disease appearance, spreading and infection to plant parts (Sharma *et al*., 2002*)*.Moreover groundnut needs a specific range of temperature for germination and healthy growth. It generally does not germinate if soil temperature is less than 15^{o}C (Prasad *et al*., 2006) while temperature of 30±4^{o}C is optimum for growth of *S. rolfsii* (Hooker, 1981; Sennoi *et al*., 2010; Buensanteai *et al*., 2012). Considering these facts minimum and maximum temperature along with humidity of experimental site were monitored over three years (2013, 2014 and 2015) during February to March (Fig.13 A, B & C). Three years observations revealed that minimum temperature above 15^{o}C and maximum temperature below 35^{o}C of Junagadh farm starts at 10th week and continues up to 11th week during summer season (January to May). Hence, we decided to screen genotypes for resistance to stem rot under field conditions during 10 – 11th week. Recommended stage of groundnut crop for screening for resistance to stem rot is 40-45days after germination (Pande *et al*,. 1994). Accordingly, groundnut genotypes were sown in the third week to have 40 days old groundnut plants ready for screening on 10 -11th week. The relative humidity during 10-11th weeks over three years remains very low in Junagadh conditions and every care was taken to ensure humidity more than 90% in the screening chambers all the time by watering plants/blocks to its maximum field capacity. Temperature inside the screening chambers was 5-10^{o}Chigher than outside temperature based on the intensity of sun shine during screening under field conditions. We observed above 40^{o}C inside the screening chambers as compared to 34 ^{o}C of outside air temperature between 11 am to 2 pm during screening period. However, around 35 ^{o}C temperature was maintained inside the screening block by opening polythene sheet and green shade net in two corners of opposite angle for 2-3 hours (Fig. 9). Opposite angles for opening of polythene sheet were decided as per same direction of wind which helps easy circulation of normal air (approximately 35^{o}C) inside the chambers. Humidity was maintained by irrigating the screening blocks in between. Desirable humidity was further ensured by watering the blocks while closing the polythene sheet and green shade net in both corners after 2-3 hours of opening. We maintained both temperature and humidity of the screening blocks in optimum level under field

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conditions which helped *S. rolfsii* to grow to its maximum potential.

Phenotyping of wild species for stem rot resistance

Wild relative of a particular crop harbors genes for resistance to biotic stresses and tolerance of abiotic stresses and are considered as important source for various desirable traits. Selected 25 accessions based on availability of seeds of 12 *Arachis* species representing four sections were screened for stem rot under pot conditions in net-house. Mortality percent at 15 days after inoculation and pod infection percent at harvest were recorded. Disease scoring was calculated using standard scale (Ghewande unpublished) and accessions were grouped into resistant, moderately resistant and susceptible ones (Table-1). None of these accessions recorded less than 10% disease scoring while, two accessions, DGR 12035 and 12047 were found resistant with mortality less than 20% (Table-2). In addition, two more accessions, DGR 11789 and 11805 were found moderately resistant with mortality less than 30%. Rest of the accessions was found susceptible with mortality ranging from 30% to 100%. Pod infection among these 25 accessions ranged from 0 - 100% although pod setting was comparatively less in net-house conditions. Pod infection in these four resistant/ moderately resistant accessions was less than 20% indicating presence of resistance in pods also. Stem rot resistant accessions DGR12035 and DGR12047 belong to two different species viz., *A. appresipila* and *A. pusilla* under Procumbantes and Heteranthae sections, respectively. On the other hand, two moderately resistant accessions though represent two different species (*A. monticola* and *A. duranensis*) but belong to the same section viz., *Arachis*. Both *A. duranensis* and *A. monticola*grouped under section *Arachis* represent the secondary gene pool while *A. appresipila* (section Procumbantes) and *A. pusilla* (section Heteranthae) represent the tertiary gene pool. All these four accessions representing four *Arachis* species are cross compatible with cultivated groundnut. Genes/alleles for resistant to stem rot from these wild accessions could be transferred into cultivated groundnut easily using conventional breeding methods or else resistant QTLs could be mapped and transferred into cultivated groundnut.

Phenotyping of segregating progenies for stem rot resistance

GG 20 is an elite Virginia groundnut cultivar widely cultivated in Gujarat, Maharastra and Rajasthan but susceptible to stem rot disease (Rakholiya and Jadeja, 2010; Bera *et al*., 2014; Thirumalaisamy *et al*., 2014). While, CS 19 is a pre-breeding genotype developed and registered by the ICAR-Directorate of Groundnut Research (Bera *et al*., 2005) and resistant to the disease (DARE/ICAR Annual report, 2003-04, Bera *et al.*, 2014, Thirumalaisamy *et al*., 2014). Polymorphism between GG 20 and CS 19 were studied using 2000 SSRs and 21 F_1 hybrids were confirmed from the cross between GG 20 as female parent and CS 19 as male parent using selected five polymorphic SSRs. Hundred and seventy-eight F_3 progenies of cross between GG 20 and CS 19 along with parents were screened under artificially inoculated field conditions and observation on mortality were recorded on 15 days after inoculation (Fig. 16 A & B) and expressed in percentage. Progenies were grouped based on their disease scoring using standard disease scale (Table -3). Three progenies (5-8, 15-1 and 15-3) were found highly resistant with less than 10% mortality followed by six, 21, and 148 progenies were resistant, moderately resistant and susceptible, respectively. These highly resistant and resistant progenies could be used in breeding groundnut varieties resistant to stem rot and in mapping genes/QTLs for resistance to stem rot in groundnut.

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Table-1 Disease scale used for grouping of groundnut genotypes based on stem rot incidence

Sl. No.	Range of mortality	Scoring
1	<10%	HR
2	10-19%	R
3	20-29%	MR
4	≥30	S

Table-2 Mortality, pod infection and scoring of wild species due to stem rot incidence

Sl No	Species	Section	ICG Accession No.	DGR Accession No.	Mortality (%)	Pod infection (%)	Scoring
1	*A. appresipila*	Procumbentes	8128	11785	50	28	S
2	*A. appresipila*	Procumbentes	8129	11786	31	13	S
3	*A. appresipila*	Procumbentes	8945	12035	14	16	R
4	*A. batizocoi*	Arachis	8210	11810	100	100	S
5	*A. batizocoi*	Arachis	8211	12030	70	0	S
6	*A. batizocoi*	Arachis	8901	12031	46	6	S
7	*A. duranansis*	Arachis	8139	11792	100	10	S
8	*A. duranansis*	Arachis	8199	11801	89	15	S
9	*A. duranansis*	Arachis	8200	11802	78	34	S
10	*A. duranansis*	Arachis	8204	11805	26	9	MR
11	*A. duranansis*	Arachis	8205	11806	60	21	S
12	*A. duranansis*	Arachis	8957	12038	78	23	S
13	*A. duranansis*	Arachis	11550	12043	76	14	S
14	*A. duranansis*	Arachis	11554	12045	70	14	S
15	*A. helodes*	Arachis	8952	12057	88	100	S
16	*A. kretschmeri*	Procumbentes	8191	12029	71	47	S
17	*A. monticola*	Arachis	8135	11789	25	19	MR
18	*A. monticola*	Arachis	8198	11800	40	14	S
19	*A. paraguariensis*	Erectoides	8141	11793	47	43	S
20	*A. paraguariensis*	Erectoides	8973	12042	87	93	S
21	*A. pusilla*	Hetranthae	Not available	12047	13	19	R
22	*A. rigoni*	Procumbentes	8186	11795	46	32	S
23	*A. rigoni*	Procumbentes	8904	12032	39	15	S

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24	*A. stenophylla*	Erectoides	8215	11811	70	50	S
25	*A. vilosa*	Arachis	8144	11794	68	23	S

Table-3 Scoring of F_3 progenies for stem rot resistance based on mortality

Progeny	**No progenies**	**Scoring**	**Mortality range**
5-8, 15-1, 15-3	3	HR	6.6 to 7.1
20-4, 1-1, 6-31, 18-7, 9-21, 2-6	6	R	11.1 to 18.7
9-17, 13-11, 2-3, 18-5, 6-28, 13-5, 4-2, 17-6, 6-29, 5-6, 9-3, 18-8, 8-5, 9-16, 21-7, 12-5, 13-6, 12-4, 17-10, 9-13, 18-6	21	MR	20.0 to 29.4
11-2, 21-4, 1-14,19-6, 20-1, 1-5, 22-6, 1-2, 1-11, 6-5, 11-3, 13-1, 22-14, 3-5, 6-40, 9-4, 17-12, 21-1, 22-13, 1-13, 6-12, 6-15, 6-32, 17-8, 18-2, 9-14, 12-1, 12-2, 12-3, 15-4, 21-3, 2-12, 6-35, 8-7, 9-19, 19-9, 22-17, 9-18, 15-2, 4-1, 6-17, 9-20, 14-11, 15-17, 18-9, 6-10, 6-25, 8-3, 12-9, 13-7, 15-20, 17-7, 7-1, 19-7, 21-2, 2-1, 22-15, 2-10, 3-2, 6-33, 9-8, 9-24, 14-12, 17-2, 2-4, 9-25, 6-4, 7-4, 17-3, 6-18, 6-24, 17-4, 21-6, 22-3, 8-1, 8-9, 14-7, 21-5, 8-2, 14-4, 3-7, 9-22, 6-41, 22-12, 5-5, 18-4, 1-12, 6-3, 6-26, 8-6, 11-5,11-9, 13-8, 14-10, 17-13, 22-11, 6-9, 6-20, 11-6, 15-7, 22-4, 3-1, 12-7, 6-13, 15-6, 4-3, 19-1, 22-7, 6-21, 15-15, 9-15, 2-13, 6-8, 2-14, 6-34, 7-2, 14-6, 18-1, 19-4, 9-2, 9-7, 11-4, 12-6, 22-2, 13-2, 6-39, 15-12, 3-8, 9-5, 16-8, 17-1, 3-3, 6-37, 15-21, 16-9, 9-9, 17-11, 9-6, 6-22, 20-2, 22-9, 5-4, 17-9, 16-3, 3-4, 5-2, 5-3, 16-2	148	S	30.0 to 93.3
CS-19		HR	9.0
GG-20		S	65.0

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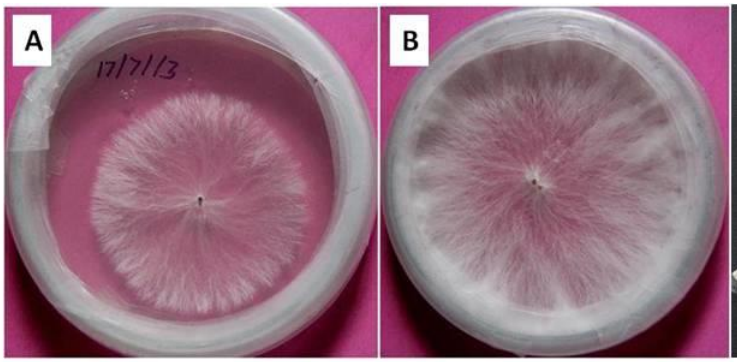

Fig.1 Sclerotium of *Sclerotium rolfsii* sub-cultured in PDA medium, A = Single sclerotium started producing of mycelium and B = Mycelium covered entire petri dish

Fig.2 *Sclerotium rolfsii* is being mass multiplied on sorghum grain in sterile poly pack

Fig.3 Thirty-five days old groundnut crop sown in lines before mulching

Fig.4 Screening block mulched with groundnut fodder

Fig.5 Screening block inoculated with fungus grown on sorghum grain

Fig.6 Rectangular shaped structure is placed on the screening block

Fig.7 Screening block covered with polythene sheet

Fig.8 Screening block covered with polythene sheet as well as green-shade net

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Fig.9 Polythene sheet and green-shade net opened in the corner

Fig.10 Wild *Arachis* species grown in Cement pot

Fig.11 Pot is inoculated with *S. rolfsii* multiplied in Sorghum

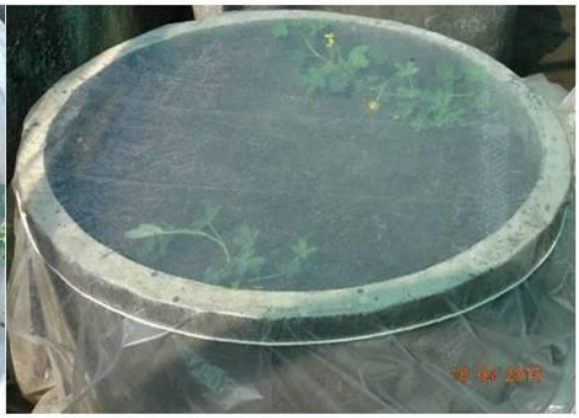

Fig.12 Pot is covered with polythene sheet followed by inoculation and watering

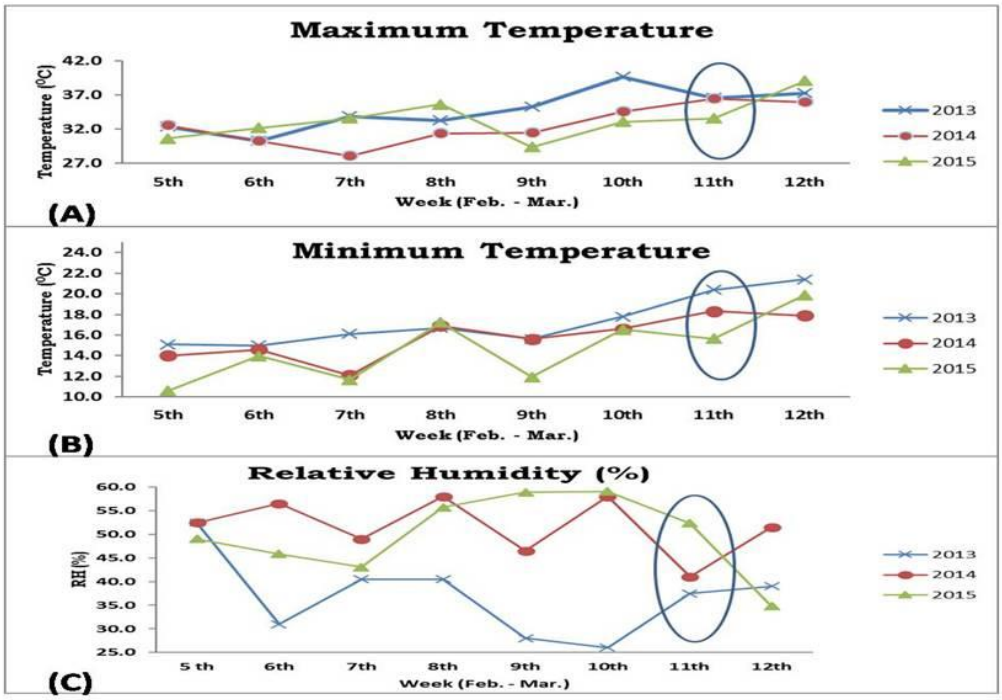

Fig.13 Weather parameters of Junagadh Farm, Gujarat during screening period; A =maximum temperature over three years, B =minimum temperature over three years, C=relative humidity over three years

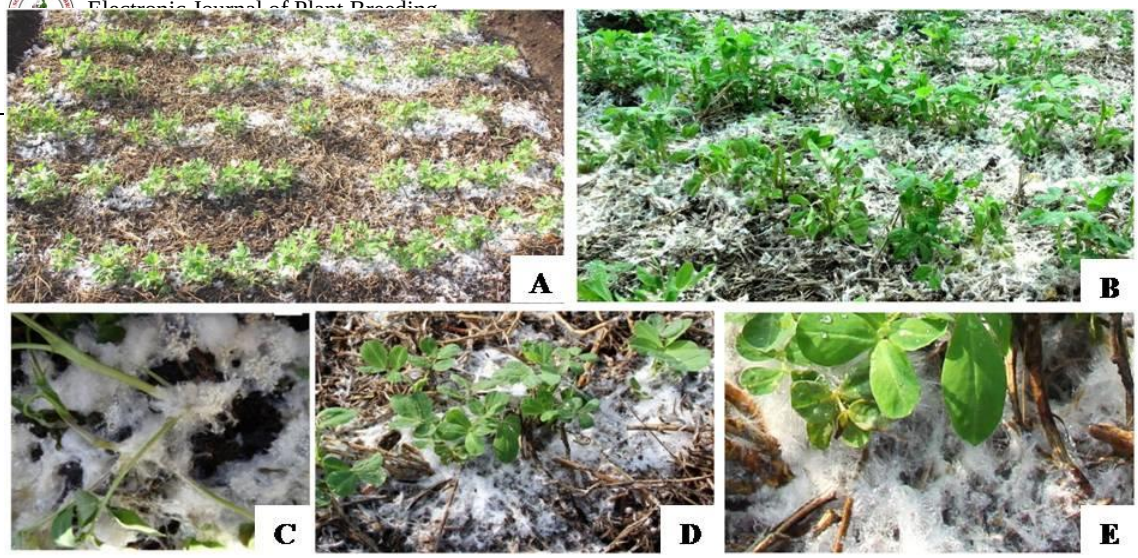

Fig.14 Huge growth of fungus forming white mat on the screening block ensuring high disease pressure under field conditions; A = Profuse growth of *S. rolfsii* on groundnut fodder after 48 hours of inoculation,B =Luxurious growth of mycelia of *S. rolfsii* after 96 hours of inoculation, C, D & E = close up view of groundnut plant covered with mycelium of the fungus

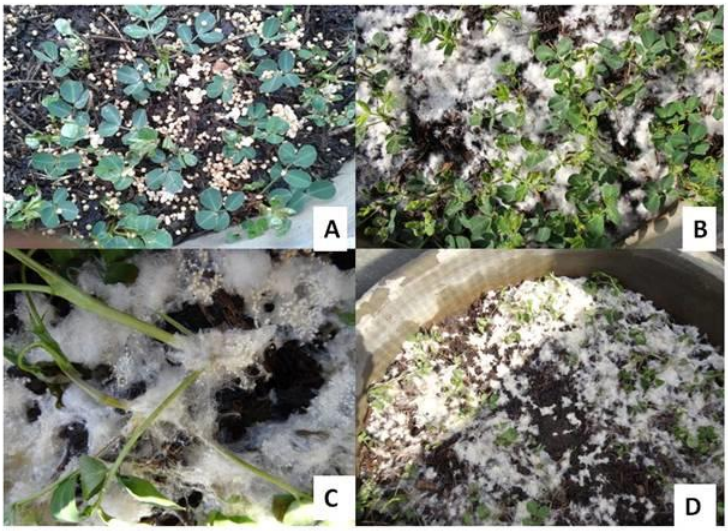

Fig.15 Screening of wild *Arachis* accessions for resistant to stem rot under small concrete pots; A = Forty days old groundnut plants are being inoculated with *Sclerotium rolfsii* multiplied in sorghum grain, B = Huge mycelium of *Sclerotium rolfsii* grown 24 hours after inoculation, C = Mycelium of *Sclerotium rolfsii* infected the plant and forming fresh sclerotium, D = wilting of groundnut plants due to infection of *Sclerotium rolfsii*.

Fig.16 Wilting and drying of plants under high disease pressure; A=Wilting of groundnut plants after 96 hours of inoculation, B= Groundnut plants died due to infection of *S. rolfsii* after 15days of inoculation