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Cultural, Morphological Characterization and Aggressiveness of *Alternaria alternata* Causing Leaf Spot of Soybean

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ABSTRACT

Background: Alternaria leaf spot caused by *Alternaria alternata* is one of the most important and destructive disease of soybean causing severe yield loss in all soybean growing areas of southern and eastern part of Rajasthan. Successful management of Alternaria leaf spot is mainly dependent on accurate and efficient detection of pathogen, amount of genetic and pathogenic variability present in pathogen population. The main reason for frequent "breakdown" of effective resistance is the variability that exists in the pathogen population, which necessitates a continual replacement of cultivars due to disease susceptibility.

Methods: The twelve fungal isolates randomly were collected from six districts of major soybean growing part of Rajasthan *i.e.,* Udaipur, Chittorgarh, Pratapgarh, Kota, Baran and Jhalawar. The culture was purified single spore techniques. These were then further compared among each other for any variations in cultural characters, colour of colonies, Growth rate, conidial morphology and pathogenic variability.

Result: Twelve different isolates of *A. alternata* were obtained in pure culture and characterized for cultural, morphological variation and aggressiveness of this fungus varied in their cultural characters, colour of colonies, growth rate of isolates, conidial morphology and isolates also exhibited variations in incubation period, latent period, number and size of lesions were produced.

Key words: *A. alternata*, Aggressiveness, Cultural, Morphological, Variation, Soybean.

INTRODUCTION

Soybean is one of the most important oil seed crops grown in India with high amount of protein and oil content in it. Among 130 diseases observed at various stages of soybean crop growth as globally, 35 diseases are economically important under different agro-conditions of India, out of which about 13 are transmitted through seed (Gupta, 2004). However, huge crop losses mainly incurred by fungal diseases, which impair the quality and yield. Alternaria leaf spot of soybean is common in Illinois during the late growing season (Chamberlain, 2011). In India, Shrivastva and Gupta (2001) have been reported leaf spot disease of soybean caused by *A. alternata.* Disease incidences up to 30% of *A. alternata* infecting soybean have been reported from Turkey and infected plants displayed necrotic, circular to oval and dark brown spots on the upper surfaces of the lower leaves Ustun *et al.* (2019). On foliage, the disease symptoms development of brown necrotic spots with concentric rings and yellow halo, large necrotic lesions that eventually coalesce and consume the entire leaf in advanced stages. Infected leaves eventually dry out and drop prematurely. The disease-infected seeds are small, shrivelled and characterized by dark irregular spreading sunken area Bhosale *et al*. (2014). One of the significant aspects of biology of an organism is the morphological and physiological characters of an individual within a species, which are not fixed. The variability is conventional phenomenon fungus of *A. alternata* and considerable range of variation in conidium morphology observed as change in spore size and shape, septation, colour, ornamentation, mycelial growth, sporulation and pathogenicity. The genetic makeup of a pathogen population is determined by the evolutionary history of that population. It is assumed that genetic makeup information also gives an idea of the evolutionary potential of pathogen populations in the future (Oguz and Karakaya, 2021). The pathogen shows variations in the pathogenic potential (virulence) and physiological functions that support its survival and perpetuation in different environmental conditions. These variations may be largely due to genetic factors that may be conditioned by environmental factors. The existence of strains, races or biotypes may be recognized by differences in virulence of the isolates on differential species or cultivars. Therefore,

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the aim of the study to identify the *Alternaria* species associated with leaf spot disease based on cultural and morphological characteristics and to reveal their aggressiveness on soybean.

MATERIALS AND METHODS

Isolation, purification and identification of the pathogen

The twelve fungal isolates randomly were collected from six districts of major soybean growing part of Rajasthan *i.e.*, Udaipur, Chittorgarh, Pratapgarh, Kota, Baran and Jhalawar and designated as UDP Aa-1, UDP Aa-2, JHA Aa-1, JHA Aa-2, PRA Aa-1, PRA Aa-2, CHI Aa-1, CHI Aa-2, BAR Aa-1, BAR Aa-2, KOT Aa-1 and KOT Aa-2. The culture was purified single spore techniques described by Sofi *et al.* (2013). Pathogenicity test was done according to Koch's postulates for all twelve isolates during *Kharif* 2018 at Rajasthan College of Agriculture, Udaipur. Based on cultural, morphological and pathogenic characteristics, the isolates identified as *Alternaria alternata* for confirmed the identity of this isolates send Indian Type Culture Collection (ITCC), Division of Plant Pathology, IARI, New Delhi (The ITCC ID No. 10.810.18, 2018).

Cultural variation

For cultural variation, twelve isolates of the pathogen grown on PDA to observe their growth pattern. The 5 mm discs of pure culture of isolates inoculated at the centre of the pre poured Petri plates from 7 days old actively growing culture. All inoculated plates incubated at temperature of 26±1°C in BOD incubator and each isolate replicated five times. The growth rate was measured and colony characters, pigmentation, growth habit and sporulation measured after 24 hours of incubation (Kumar and Choudhary, 2006).

Morphological variation

To purify *A. alternata* culture, a conidial suspension was prepared in sterilized distilled water from 7 days old culture on PDA and flooded on 2% plain agar in Petri plates. The excess suspension drained out and the Petri plates then incubated in invert position at 26±1°C. After eight hours a single germinating spore was marked with the help of dummy objective and transfer individually with a piece of plain agar medium to PDA slants by inoculating needle under aseptic conditions. These monoconidial isolates maintained on PDA slants further used to study of spore morphology as described by Boedo *et al.* (2012). The observation on variation in conidial morphology of twelve isolates of *A. alternata* recorded with the help of ocular and stage micrometre (Gupta and Pandey, 2013).

Assessment of *A. alternata* aggressiveness

Aggressiveness is the genetic character of fungi, which may vary amongst test isolates. Healthy seeds of soybean variety RKS-24 were surface sterilized then sown in sand: soil: FYM (3:1:1) mixture keeping three replications for each isolate in completely randomized design having 10-15 plants in each pot. The plant leaves, stem and branches of six weeks old infected plants, randomly were selected and spore of fungi from infected plant part separated gently by delicate brush and suspended in seven days old culture of each isolate of *A. alternata*. Simultaneously, un-inoculated check was maintained by spraying sterilized distilled water on plants. The inoculated plants observed daily to record the incubation period for the disease development. Percent disease index calculated formula with help of disease rating scale (0-5) given by Sangeetha and Siddaramaiah, (2007).

Standard disease rating scale

Rating scale	Description of the symptom
0	Leaves free from infection
1	Small irregular spots covering <5% leaf area
2	Small irregular brown spots with concentric rings covering 5.1-10% leaf area
3	Lesions enlarge, irregular brown with concentric rings covering 10.1-25% leaf area
4	Lesions coalease to form irregular and appears as a typical leaf spotting symptom covering 25.1-50% leaf area
5	Lesions coalease to form irregular and appears as a typical leaf spotting symptom covering >50% leaf area

Numbers of plants in each score were recorded and the PDI in each plot was determined as:

$$PDI = \frac{n\times1+n\times2+n\times3+n\times4+n\times5}{N} \times \frac{\text{Maximum disease score (5)}}{100}$$

Where,
n = Number of plants in each score.
1-5 = Disease score.
N = Total number of plants under observation.

RESULTS AND DISCUSSION

Cultural variation

Characterization of the isolates indicated that most of the cultures were light dark or light brown or velvety in colour with light or dark brown pigmentation and regular or irregular growth pattern with smooth white margin steel grey in centre and sporulation were also varied for the test isolates. The colony diameter and amount of sporulation ranged from 72.4 mm to 87.2 mm and 9.5×10^3 to 15.9×10^3 /mm^2 medium) at seven days after incubation at 26±1°C in 90 mm Petri plates. Maximum radial growth and amount of sporulation was recorded by UDP Aa-1 (87.2 mm, 15.9×10^3 /mm^2 medium) followed by UDP Aa-2 (85.9 mm, 15.0 10^3 /mm^2 medium). Least mycelial growth and amount of sporulation was recorded by KOT Aa-1 (73.7 mm, 10.2×10^3 /mm^2 medium) and KOT Aa-2 (72.4 mm, 9.5×10^3 /mm^2 medium) (Table 1, Fig 1).

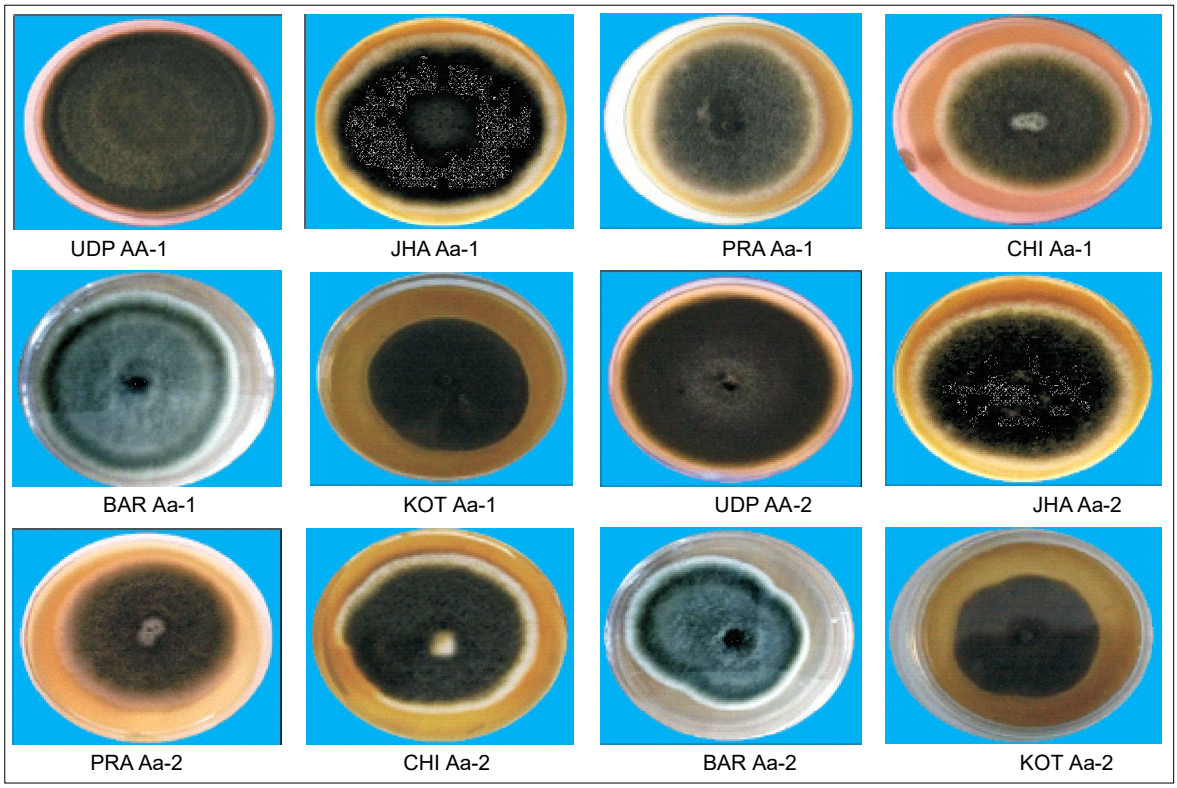

Fig 1: Cultural variation among twelve isolates of *A. alternata* on potato dextrose agar media.

Table 1: Radial growth and cultural characters of twelve isolates of *A. alternata*on PDA.

Isolates	Diameter* (mm) 7th days	Sporulation** (×10³/ mm² medium)	Growth characters and colony colour
UDP Aa-1	87.2	15.9	Felty velvety suppressed growth, dull black or steel grey in centre, very fast mycelial growth, clear zonation present with smooth margin.
UDP Aa-2	85.9	15.0	Aerial feltymycelial growth, dark black or whitish grey in centre, zonation present with smoothwhite margin.
JHA Aa-1	84.6	14.8	Felty, velvety suppressed growth, compact black or dark grey in centre, clear zonation present with light brown at periphery.
JHA Aa-2	83.3	13.9	Aerial, fletymycelial growth, light black in colour, zonation present with dull white towards margin.
PRA Aa-1	78.3	12.0	Slightly blackish with grey and slow mycelia growth, zonation present with regular white margin.
PRA Aa-2	77.5	11.7	Light black with brown colour, at centre white mycelial growth and slow growing, zonation present and regular light brown at periphery.
CHI Aa-1	76.9	11.5	Light black coloured, light brown in centre, zonation present medium mycelial growth with smooth white margin.
CHI Aa-2	75.7	11.3	Black coloured, zonation present, flat mycelial growth with smooth white margin but sometime irregular.
BAR Aa-1	75.2	11.1	Cottony velvety submerged uniform mycelial growth, light white colour without zonation with regular white margin.
BAR Aa-2	74.0	10.7	Felty velvety cottony and light green mycelial growth, light zonation present and dull white margin.
KOT Aa-1	73.7	10.2	Dense, compact blackish growth, concentric rings not clear, without zonation.
KOT Aa-2	72.4	9.5	Feltyflocose blackish submerged growth, without zonation, concentric ring not clear.
CD at 5%	5.66	0.99	
CV (%)	4.27	4.82	

*Mean of five replications; **Mean of five replications, three from each of the plate.

In accordance with the current results by Chethana *et al.* (2018) reported that cultural variation of fifty six isolates of *Alternaria* spp. exhibited ash, ashy black, ashy white, ashy green and blackish green colour. Rajender *et al.* (2013) observed that variation in colony diameter varied from 21.0 mm to 42.0 mm and abundant sporulation (12 × 10^4 spores ml^{-1}) in isolates of *A. helianthin*. Our results are also collaborated with Nikam *et al.* (2015) and Mohsin *et al.* (2016).

Morphological variation

In respect of morphological characteristics, the isolates of *A. alternata* showed variation in conidia production, shape and size of conidia (with beak and without beak). The conidial length and width ranged from 18-27 × 6-9 µm (with beak) and 11-14 × 6-9 µm (without beak) and 36-46 × 14-19 µm (with beak) and 32-40 × 12-15 µm (without beak). The minimum conidial length and width recorded by UDP Aa-1, most obclavate rarely obovate, long beak without septation, thick walled, more rounded at tips, dark olivaceous brown with darker septation and measuring 18-27 × 6-9 µm (with beak) and 11-14 × 6-9 µm (without beak) followed by UDP Aa-2. Obclavate, solitary, oblique, rarely muriform, smooth walled, small in size, slightly straight with shorten beak, more rounded at tips, pale olivaceous, brown with dark septation and measuring 20-24 × 7-10 µm (with beak) and 13-16 × 7-10 µm (without beak). The minimum conidial length and width recorded by KOT Aa-1, solitary most obclavate rarely obovate, slight flexuous, small in size, long beak without septation, thick walled more rounded at the tips, tapering to beak, smooth, pale olivacious brown with dark brown septation and measuring 30-38 ×14-17 µm (with beak) and 16-33 × 11-14 µm (without beak). KOT Aa-2, solitary, obovate, often rostrate, simple straight, constricted at the septa, pale olivacious brown with darker septation and measuring 36-46 × 14-19 µm (with beak) and 32-40 × 12-15 µm (without beak) (Table 2, Fig 2). The results of the study also concur with the finding of Devi *et al.* (2016) recorded the maximum conidial length (62.16 µm), width (15.60 µm), beak length (24.50 µm) and maximum number of conidial cells (3-8) in *A. helianthi*. Rajender *et al.* (2013) recorded the average range of conidial length 124.2 to 158.4 µm (with beak) and width 30.7 to 40.1 µm (without beak) of *A. helianthi*, respectively. Furthermore, similar results were also observed by Jankar *et al.* (2018), Reddy *et al.* (2019) and Aung *et al.* (2020).

Assessment of *A. alternata* aggressiveness

Study conducted to characterize in pathogenic variation of *A. alternata* isolates from distinct geographical areas of Rajasthan showed that significant difference exists in host response. The isolates UDP Aa-1 was found to be highly aggressive on soybean cv. RKS-24 under artificial inoculated conditions, showing 65.2% disease incidence followed by UDP Aa-2 (63.8%), JHA Aa-1 (56.3%), JHA Aa-2 (54.5%), PRA Aa-1 (49.2%), PRA Aa-2 (48.3%), CHI

Table 2: Variation in conidial morphology of twelve isolates of *A. alternata* on PDA.

Isolates	Conidial morphology with beak				Conidial morphology without beak			
	Length (µm)		Width (µm)		Length (µm)		Width (µm)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
UDP Aa-1	22.3±0.89	18-27	7.3±0.87	6-9	14.6±0.42	11-14	6.3±0.24	6-9
UDP Aa-2	24.7±0.69	20-24	8.3±0.61	7-10	15.3±0.72	13-16	7.6±0.34	7-10
JHA Aa-1	25.8±1.27	23-29	9.5±0.77	8-11	18.2±0.61	15-18	8.2±0.42	7-10
JHA Aa-2	27.6±1.60	24-30	9.8±1.00	10-12	20.9±1.67	16-20	8.6±0.69	8-11
PRA Aa-1	28.1±1.61	25-31	10.0±0.58	9-12	21.5±1.27	17-21	9.6±0.46	9-11
PRA Aa-2	30.7±1.39	23-29	10.3±0.76	10-13	22.6±1.25	18-24	10.2±0.52	9-12
CHI Aa-1	32.3±1.90	29-35	10.7±0.63	11-14	22.8±1.24	23-29	10.9±0.62	10-12
CHI Aa-2	33.6±1.70	30-39	11.5±0.53	12-15	23.9±1.22	26-33	11.0±0.63	10-12
BAR Aa-1	34.0±2.37	29-36	12.0±0.70	12-15	26.4±1.32	19-25	11.2±0.60	10-13
BAR Aa-2	36.8±1.75	34-42	12.2±0.58	13-16	27.8±1.51	20-26	12.3±0.70	10-13
KOT Aa-1	38.2±2.20	30-38	12.6±0.60	14-17	28.3±2.20	16-33	12.7±0.70	11-14
KOT Aa-2	41.6±2.45	36-46	13.4±0.65	14-19	32.4±2.00	32-40	13.1±0.67	12-15
CD at 5%	1.01		0.33		0.78		0.33	
CV (%)	2.03		1.99		2.14		2.07	

*Mean no. of 50 conidia and ± S.D. of mean value.

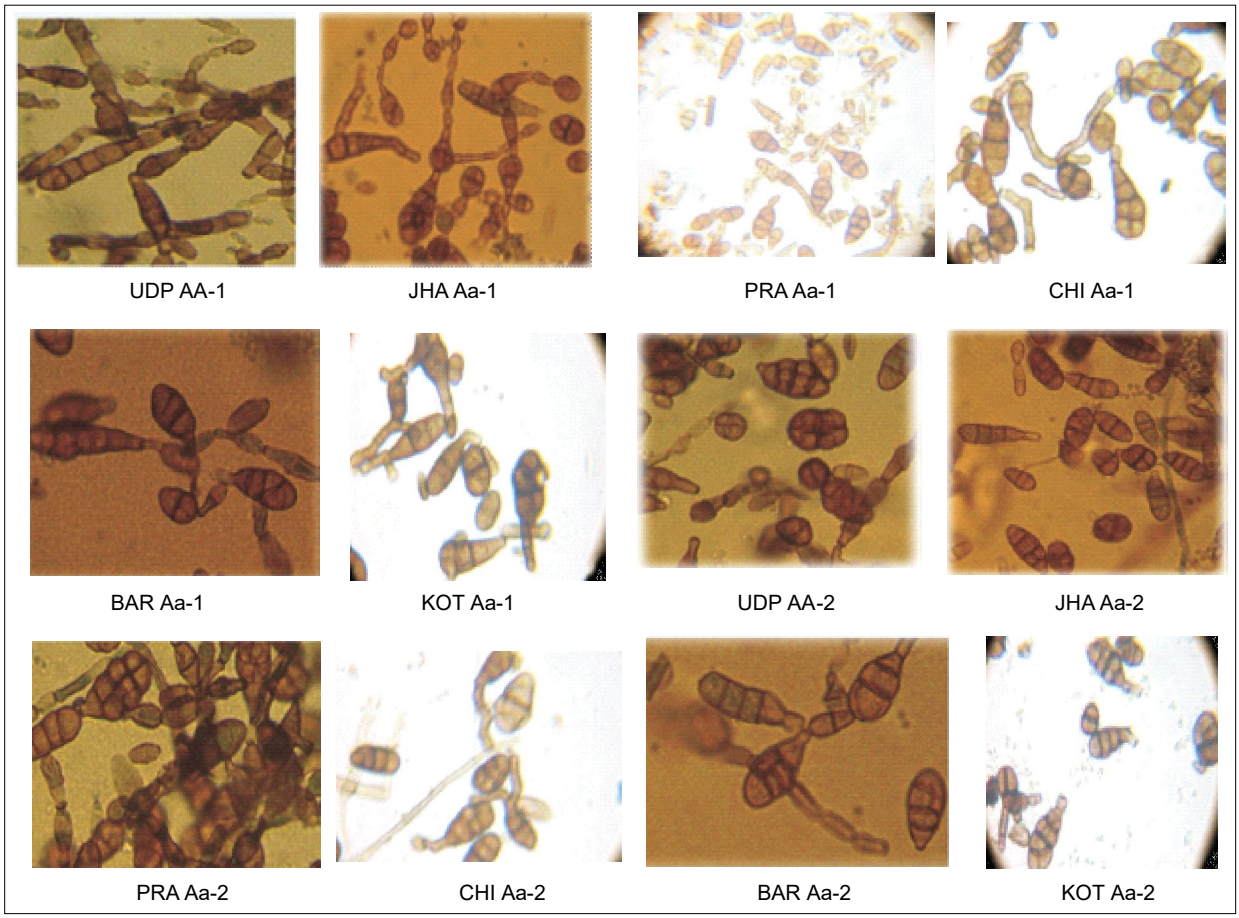

Fig 2: Conidial Morphology among twelve isolates of *A. alternata* on potato dextrose agar media.

Table 3: Latent period (after 24 hrs. of inoculation) and PDI of different isolates of caused by *A. alternata* on pot-grown plants of soybean.

IIsolates	Latent period (hrs.)	Per cent disease index (PDI*)
UDP Aa-1	35	65.2 (53.8)
UDP Aa-2	36	63.8 (53.0)
JHA Aa-1	40	56.3 (48.6)
JHA Aa-2	42	54.5 (47.5)
PRA Aa-1	46	49.2 (44.5)
PRA Aa-2	47	48.3 (44.0)
CHI Aa-1	51	42.2 (40.5)
CHI Aa-2	51	40.0 (39.2)
BAR Aa-1	55	34.3 (35.8)
BAR Aa-2	57	33.7 (35.4)
KOT Aa-1	61	27.2 (31.4)
KOT Aa-2	63	26.6 (31.0)
CD at 5%	2.44	3.24
CV%	2.97	4.25

*Mean of three replications, Figures in parentheses are arcsine √per cent transformed values.

Aa-1 (42.2%), CHI Aa-2 (40.0%), BAR Aa-1 (34.3%), BAR Aa-2 (33.7%), KOT Aa-1 (27.2%) and KOT Aa-2 (26.6%). The initial chlorotic/pin point necrotic spots of Alternaria leaf spot started appearing in 35.00 hrs after inoculation. The seedlings grown applying sterilized distilled water without inoculation did not produce any leaf spotted symptoms and grew healthy (Table 3, Fig 3). The way in which fungal populations depends primarily on the type of genetic variability available and also variation in population structure of the pathogen becomes the major reason for resistance break down of many high yielding resistant varieties. The similar pathogenic variability results were obtained by Mohsin *et al.* (2016) reported that test isolates of *A. porri* also exhibited variations in size of the lesions (2.77 to 7.55 mm) produced on onion leaves. Jankar *et al.* (2018) reported that all isolates of are pathogenic, while Isolate Aa-5 (Nagpur) was the most virulent isolate and Aa-4 (Akola) was the less virulent isolate of *A. alternata* causing fruit rot of chilli. Our results are corroborated with Loganathan *et al.* (2014) that observed pathogenic variability among seventeen isolates of *Alternaria* spp. infecting tomato in northern India.

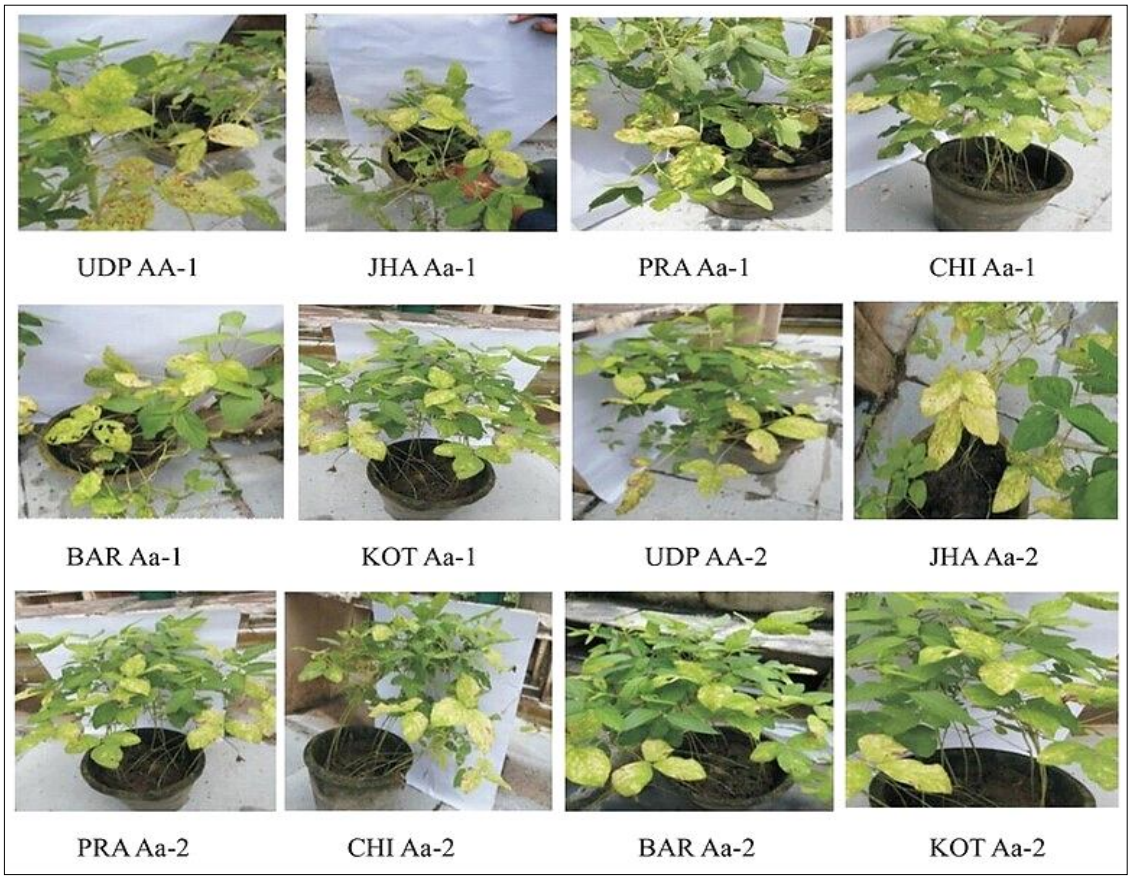

Fig 3: Symptoms on leaves of susceptible land race (RKS-24) of soybean inoculated with different isolated of *A. alternata* in pot condition.

CONCLUSION

Based on these experimental results that cultural, morphological and pathogenic variability of *A. alternata* infecting soybean crop in major growing part of Rajasthan. The presence of *A. alternata* in virulent new pathotypes with the introduction of new type of variety and hybrids to our crops and also with excessive use of chemicals. Rapid and accurate detection of new virulence will help to formulate strategy for developing resistant cultivars in particular region and will also provide a base for breeding cultivars with durable resistance or designing strategies for the long-term management of major diseases through chemicals. Thus, variation in population structure of the pathogen becomes the major reason for resistance break down of many high yielding resistant varieties and evolution of pathogenic strains offering resistance to popularly used fungicides.

Conflict of interest

All authors declared that there is no conflict of interest.

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