

Original Research Article

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## Improved Detached Leaf Assay for Rapid Screening against Late Leaf Spot in Groundnut

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### ABSTRACT

#### Keywords

Arachis, Late leaf spot, Detached leaf assay, Latent period, Per cent leaf area damaged, Host plant resistance

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The use of resistant cultivars is the most economical and effective method to control late leaf spot (*Phaeoisariopsis personata*) in groundnut. In the process of breeding for host plant resistance, the plant material is evaluated in the hot spot location under field conditions. But this process of evaluating the whole crop cycle demands lot of labour and time, the disease expression fluctuates with environmental conditions. Here, we present the improved detached leaf assay method for screening groundnut lines under controlled conditions. Three genotypes of groundnut viz., GPBD 4, ICGV 13193 and TMV 2 were screened against late leaf spot. Based on visual observation of incubation period, percentage of leaf area damaged, lesion diameter, latent period and defoliation percentage were recorded. The varieties GPBD 4 and TMV 2 consistently showed lowest and highest disease intensities, respectively. While the introgression line ICGV 13193 showed moderate resistance. This methodology may allow the detection of sources of resistance that specifically involve one or more components of resistance for defence mechanisms against LLS. When combined with field assessment, this methodology should also enable to quantitatively assess the relative role of each components of resistance to LLS. Therefore, detached leaf assay can be used to screen groundnut genotypes against late leaf spot.

### Introduction

Among biotic constraints that limit groundnut yield, foliar fungal diseases are important globally. *Cercospora* or tikka leaf spots (early and late leaf spot) are the most important fungal diseases of groundnut. Late leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk. and M.A. Curtis) van Arx. is more prevalent during the rainy season in all groundnut-growing areas of India. It starts

around mid-August when the fungal spores germinate and penetrate peanut leaves through stomata located on the abaxial surface.

Round-shaped black lesions become visible within 1 week of fungal germination. Since the spores are deposited in the soil, LLS disease usually starts from the bottom layer of the plant canopy and moves upward. As the fungal lesions enlarge, coalescence of lesions is often observed in highly susceptible lines

(Gill, 2013). Sporulation occurs 20 to 30 days after infection. Secondary infection from freshly produced spores is common in the long peanut growing season. Leaf spot affects by reducing the available photosynthetic area, localized damage of leaf tissues and defoliation. Highly susceptible lines can lose all their leaves one month prior to maturity. The necrotic area and the defoliation induced by LLS can cause 30-70% yield penalty in susceptible cultivars (Bourgeois *et al.*, 1991 and Singh *et al.*, 2011).

In resistance breeding program, evaluation for the host resistance needs to be performed with accuracy which is conducted either in greenhouse and/or field conditions using whole seedlings or adult plants screening, respectively. Field screening can be conducted only during crop season and there will be masking effect of one gene over other.

Moreover, the fluctuating environmental conditions, especially temperature and humidity, greatly influence the disease progress. In field environment, different unrelated pathogens and/or various races of the same pathogen may co-infect the plants, which can complicate the assessment procedure.

The alternative to field-based assessment is to carry out evaluation in a controlled environment (laboratory experiments) where whole-plant screening can be done. However, whole-plant screening also has its limitations, including a requirement for a spacious controlled environment glasshouse and also requires relatively high bulks of inoculum. So, a screening technique mitigating the constraints of seedling and adult plant screening is required during pre-breeding of resistant varieties. The advanced detached plant part screening assay allows better control over temperature and humidity, and the use of larger number of replicates with lesser

quantity of inoculum and can retain the healthy plant for further studies.

Detached plant part screening assay, a rapid screening technique that screens materials under controlled condition has been used in many crops. Detached leaves have been used to screen plant material against many diseases like early leaf spot of groundnut (Ricker *et al.*, 1985), anthracnose in white beans (Tu, 1986), *Colletotrichum* of Arabidopsis (Liu *et al.*, 2007), crown rust of oat (Jackson *et al.*, 2008), late leaf spot of groundnut (Janila *et al.*, 2013), powdery mildew in pea (Azmat *et al.*, 2013), leaf rust of wheat (Boydton *et al.*, 2013) and Net Blotch of Barley (El-Mor *et al.*, 2018)

The objective of this study was to examine the feasibility of using improved detached leaf assay for disease reaction assessment of breeding lines. Our approach was to compare results for inoculated detached leaves of different crop growth stages.

## **Materials and Methods**

### **Plant material for detached leaf assay**

The genotypes *viz.*, GPBD 4 (resistant check), ICGV 13193 (moderate resistant) and a susceptible control TMV 2 were selected for the late leaf spot screening study. Seeds of these genotypes were sown in field condition in the experimental plots of Alfisols (Alfisol-Patancheru Soil Series; UdicRhodustolf) at ICRISAT, Patancheru, India (at 17.53° N latitude and 78.27° E, 545 m.a.s.l.) during post-rainy season of 2018. The fully expanded quadrifoliate leaves (third or fourth from top) from 45 and 70 days old plants were excised through pulvinus and screened for LLS resistance in two separate experiments designated as set 1 and set 2 respectively. The excised leaves were washed with deionised water and used as an explant.

### **Experimental design, screening techniques, data recording and analysis**

Each test was carried out in two replicates in a Complete Randomized Design (CRD). The mixture of sand: vermiculite mixture (1:1 v/v) was prepared and steam sterilized at 15 lbs pressure and 121° C for 1 h in two cycles of autoclave. The excised explants from all three genotypes were planted in sterile culture (1.5 cm thick sand) in plastic trays (40 x 30 cm) and transferred to dew chamber. Immediately after planting the detached leaves in the tray were sprayed with LLS inoculum (30,000 conidia ml<sup>-1</sup>). The dew chamber conditions were maintained at a temperature of 24° C and 85% relative humidity. Further the trays were covered with transparent plastic sheets to maintain suitable conditions for disease development. Water was sprayed on the culture, leaves and the sheets once daily up to 8 days. Disease development was monitored every alternate day from 5 to 40 days after inoculation (DAI). The following parameters were studied for the components of resistance and histological parameters.

#### **Components of resistance to LLS**

Data on the following parameters were recorded:

1. The incubation period (IP): IP is defined as days from inoculation to appearance of the first lesion. It is recorded on each leaf every alternate day from 5 days to 40 DAI.
2. Latent period (LP): LP is defined as days from inoculation to the appearance of the first sporulating lesion. It is recorded on each leaf every alternate day from 5 to 40 DAI.
3. Leaf area damage (LAD): The percent LAD was assessed by comparing the leaves with diagrams depicting leaves with known

percentage of their areas affected. It was measured at 40 DAI.

4. Lesion diameter (LD): LD is the average diameter of five randomly selected lesions on each leaflet. It was measured at 40 DAI using vernier caliper under 20 × magnifying lens and the data was recorded in mm.
5. Defoliation percentage (DP): Percent leaf defoliation and disease scores were recorded at 40 DAI. The percent defoliation was calculated based on the total number of leaves and number of leaves defoliated.

The disease score for LLS were recorded at 20 and 40 DAI, referring modified 1-9 scale, as given by Subrahmanyam *et al.*, (1995). For detached leaf assay, the results were differentiated on the basis of appearance of symptoms and symptom-free leaflets. All the statistical tests and parameters were conducted using Microsoft Excel (version, 2016).

### **Results and Discussion**

The results on screening of fully expanded excised quadrifoliate leaves provided a clear-cut differentiation between susceptible, resistant and moderately resistant cultivars based on artificial inoculations with spore concentration higher than 30,000 conidia ml<sup>-1</sup>. Since disease severity in the susceptible cultivar increased with duration, the differentiation became more evident. The modified composition of culture medium with mixture of sand:vermiculite mixture (1:1 v/v) enabled maintenance of optimum moisture and humidity (85 %) with minimum contamination of saprophytes and other fungus.

The experimental results of Set 1 and Set 2 are presented in Table 1 and 2, respectively. This showed that the disease severity and incidence

were generally not affected by leaflet age; except in the case of percent leaf defoliation. The infection was identified by dark necrotic spots to the area of the abaxial surface of leaf

and the appearance of black spores on the centre of necrotic lesion was recorded as latent period.

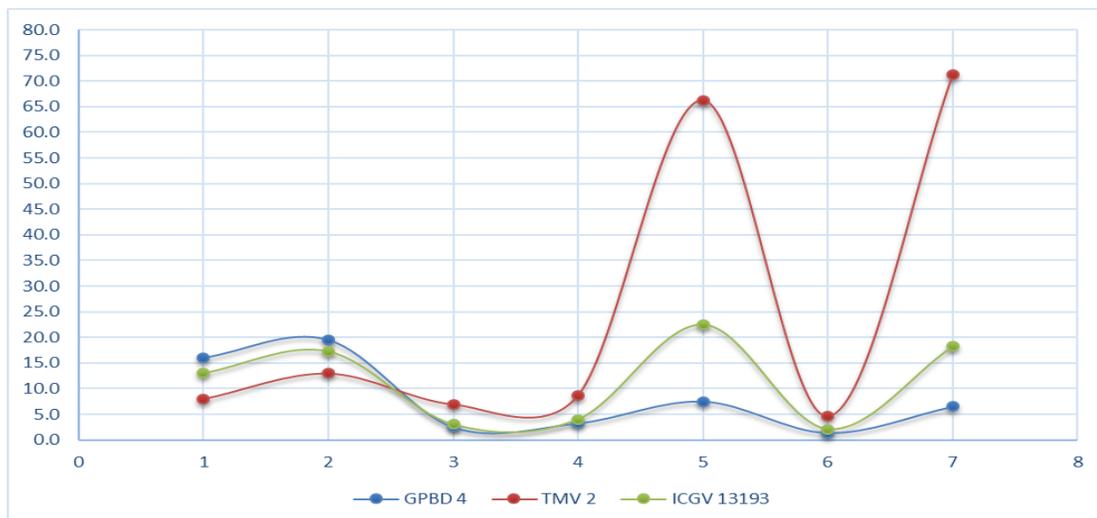
**Table.1** Response of groundnut genotypes to late leaf spot screening using detached leaf assay under controlled conditions in two independent experiments at 45 and 70 days old crop stage

Set 1: Dew chamber experiment at 45 days old excised leaves							
	IP	LP	LLS_20 DAI	LLS_40 DAI	% LAD	LD (mm)	% LDF
GPBD 4	16.5	19.5	2.5	3.0	6.0	1.4	6.0
TMV 2	7.5	13.0	6.5	8.5	62.5	4.6	67.5
ICGV 13193	12.5	17.0	3.0	4.0	22.5	2.2	17.5
Sem (±)	0.6	0.3	0.3	0.3	2.6	0.1	2.6
LSD 5%	3.5	1.8	1.8	1.8	15.6	0.7	15.6

Set 2: Dew chamber experiment at 45 days old excised leaves							
	IP	LP	LLS_20 DAI	LLS_40 DAI	% LAD	LD (mm)	%LDF
GPBD 4	15.5	19.5	2.5	3.5	9.0	1.4	7.0
TMV 2	8.5	13.0	7.5	9.0	70.0	4.8	75.0
ICGV 13193	13.5	17.5	3.0	4.0	22.5	2.0	19.0
Sem (±)	0.6	0.3	0.8	0.3	2.5	0.1	3.1
LSD 5%	3.5	1.8	4.6	1.8	11.0	0.5	18.6

IP: Incubation period, LP: latent period, LLS\_20 DAI: LLS score at 20 days after inoculation, LLS\_40 DAI: LLS score at 40 days after inoculation, % LAD: Per cent leaf area damaged by LLS, LD: Lesion diameter (mm), % LDF: Per cent leaf defoliation

**Fig.1** Pooled mean of experiment Set 1 and Set 2. (1) Incubation period in days, (2) latent period in days, (3) late leaf spot score at 20 DAI, (4) late leaf spot score at 40 DAI, (5) percent leaf area damaged, (6) lesion diameter in mm, and (7) percent leaf defoliation



Across three groundnut genotypes in the two experimental sets, the mean incubation and latent periods (Fig. 1) were longest in resistant cultivar GPBD 4 (16 and 19.5 DAI), moderate in ICGV 13193 (13 and 17.3 DAI) and shortest in susceptible cultivar (8 and 13 DAI) at 24<sup>o</sup> C and 85% relative humidity. The mean disease score of resistant check GPBD 4 was 2.5 and 3.3, while that of susceptible cultivar TMV 2 was 7.0 and 8.75 at 20 and 40 DAI, respectively. The cultivar ICGV 13193 had mean scores of 3 and 4 at 20 and 40 DAI, respectively and was graded as moderately resistant. The resistant cultivar GPBD 4 showed smaller lesion diameter (LD) of 1.4 mm. The moderately resistant cultivar ICGV 13193 measured 2.1 mm LD and the susceptible cultivar TMV 2 showed largest lesions with 4.7 mm LD.

The cultivar TMV 2 was severely infected with LLS exhibiting 66.3% percent leaf area damage (LAD) and the genotype ICGV 13193 was moderately infected with 22.5% LAD. The resistant genotype, GPDB 4 showed least damaged area accounting only 7.5% LAD. Percent leaf defoliation (% LDF) was the most variable parameter with least defoliation in GPBD 4 (6.5%), moderate defoliation in ICGV 13193 (18.3) and maximum in TMV 2 (18.2 %).

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The results also showed that leaflets taken from different growth stages of plants responded similarly to inoculation as they

were excised immediately after being fully expanded. The well-known resistant check, GPBD 4 showed clear resistance to LLS fungus in both the experiments while susceptible cultivar, TMV 2 was severely affected with leaf spot. The cultivar ICGV 13193 exhibited moderate resistance with a mean score of 4 in both the screening assay.

Detached leaf culture was originally described by Subrahmanyam *et al.*, (1990) as a bioassay for obligate leaf pathogens. The current study examined the feasibility of using modified detached leaflet assay for disease assessment in late leaf spot of groundnut. In earlier studies, either sand culture (Janila *et al.*, 2013) or whole plant assay (Dwivedi *et al.*, 2002) was used to screen detached leaf against LLS. But the inclusion of sand:vermiculite mixture (1:1 v/v) enabled to maintain higher humidity and proper moisture in the medium with secondary infection. The disease reaction of leaflets inoculated with artificial inoculum is determined using both disease incidence and severity values, was generally similar to the reaction of the intact plant (Dwivedi *et al.*, 2002).

The ability to quickly screen breeding lines for resistance is often limited by the amount of controlled environment space for the required number of plant lines and pathogen isolates. Low standard errors for each evaluation method set 1 and set 2 further confirm the consistency of the disease response ratings between the detached leaf. This showed that the disease severity and incidence were generally not affected by leaflet age; the exceptions to this were results for both parameters of percent leaf defoliation. Defoliation may occur due to the severe fungal infection at leaf node and further resulted in physiological senescence. Other workers using detached leaves have also reported that the cultivar GPBD 4 is resistant to LLS while TMV 2 is highly

susceptible to LLS (Dwivedi *et al.*, 2002 and Janila *et al.*, 2013). There are several advantages with the excised leaf method: (a) a plant can be used to screen for different races of a pathogen, (b) seeds of a partially resistant plant with favourable agronomic characteristics can be saved for future breeding purposes, (c) if resistance to different races of a pathogen involves two or more genes, the genetic composition of each segregating progeny of a cross can be determined, and (d) assay can be suitable in histo-pathological studies of plant.

With this method, a plant can be screened many times because screening can be done at any growth stage while they are grown in the field or in the greenhouse without affecting their growth and seed production. This method offers considerable time saving in breeding for resistance. It works well on the newly expanded leaves because they appear to have less contamination with other foliar organisms. This method would be valuable for evaluating groundnut for resistance to late leaf spot when limited quantities of seed are available in early generation breeding material and the experiment cannot be conducted in replication, e.g., F<sub>1</sub>, F<sub>2</sub> seeds.

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