Morpho-anatomical characterization of groundnut genotypes showing differential reaction to late leaf spot pathogen

R. Trivedi[1]

[1]Department of Botany, Mohanlal Sukhadia University, Udaipur, Rajasthan, India

Corresponding Author: **rohini.l.trivedi@gmail.com**

Introduction

Grain legumes are the major source of proteins for more than two billion people world-wide. Among the grain legumes, peanut or groundnut (*Arachis hypogaea* L.) is an important crop valued for its high content of protein and oil. Groundnut kernels contain about 43% oil and 25% protein. Biotic and abiotic stresses drastically limit the plant growth and crop productivity in groundnut. Of the various pathogens present, maximum damage is caused by fungi. Early leaf spot (ELS) caused by *Cercospora arachidicola* and late leaf spot (LLS) caused by *Phaeoisariopsis personata,* are amongst the major fungal diseases of groundnut. Leaf spots can cause yield losses of up to 50% worldwide (Tshilenge-Lukanda et al., 2012). Host genetic resistance against pathogens (especially foliar pathogens) can be directly associated with the leaf morpho-anatomical characteristics (Chattopadhyay et al., 2011).

The present study was undertaken to evaluate the role of micro-morphological and anatomical characters of leaf and to establish their correlation between disease tolerances in three groundnut genotypes showing differential response to LLS disease. Histopathological studies were also carried out on the LLS infected leaves of the selected genotypes.

Materials and Methods

Three groundnut genotypes ICGV-86699 (tolerant), ICGV-86590 (moderately susceptible to LLS), and TMV-2 (susceptible to LLS) were selected for the present studies. Groundnut plants of the three genotypes were grown in 15 cm diameter plastic pots in a green house in 50% sand and 50% soil with compost. One square cm block from the middle portion of lamina of the third fully expanded leaf from apex of a one month old greenhouse grown plants from each genotype was cut, for the purpose of leaf surface and anatomical studies.. Samples for leaf surface studies were fixed and cleared in fixative containing glacial acetic acid and ethanol in 3:1 volume /volume (v/v) ratio. These samples were stained with lactophenol cotton blue and observed under the compound microscope. The number of stomata and trichomes were recorded for both the surfaces. The length and diameter of stomatal aperture were determined using the micrometry technique.

For inoculum production, single lesion isolate of LLS pathogen were harvested from the field infected leaves by cyclone spore collector and stored at 4ºC. In order to make spore suspension, the spores collected were suspended in water with a little amount of Tween-20 and stirred using a magnetic stirrer. The spore concentration was adjusted as required with the help of a haemocytometer. For infection studies, healthy one month old leaves excised through the pulvinus base were washed in running water and arranged in plastic trays with their petioles buried in steam sterilized sand. Trays/leaves were watered sufficiently and covered with plastic bags with the ends sealed with cellophane tape to maintain high humidity. The trays were then kept in Percival incubators at 25ºC and 12 hours photoperiod (4000 lux) for 24 hours. Inoculation was done on the upper surface of the leaflets by placing a drop of inoculum with the help of a micro syringe and spreading it with a paintbrush. The inoculum density was maintained at 65,000 conidia/ml. The spread was allowed to dry and the trays were covered with a moist polythene bag. After the application of the spore suspension on the leaves, the trays were covered with polythene bags which were made airtight using cellophane tape. The trays were then kept in an incubator at 25ºC and 95% relative humidity with 12 hour light and 12 hour dark period. The alternate wet and dry period accelerated the growth of pathogen because of which the plastic bags were removed for four to six hours during the light period and again covered after spraying water. The inoculated portion was cut out at an interval of four hours starting from the zero hour to 48 hour. It was then processed and stained as described earlier, for initial infection studies. For samples of histopathological studies, the same region of leaf was taken, but after

48 hours of inoculation with the late leaf pathogen and subsequent sampling was done at 12 hour interval. The samples for anatomical and histopathological studies were fixed in small glass vials containing the fixative (2% glutaraldehyde and formaldehyde solution) for 24 hrs. After fixation, the fixing solution was decanted and replaced with different grades of ethanol (50%, 70%, 90% and 99.9%). The samples were then kept in fixing solution for overnight in each grade of ethanol. After dehydration, absolute alcohol was decanted and replaced with an intermediate solution. Samples were first soaked in the intermediate solution for 48 hrs and then in the filtration solution and this continued until the tissue appeared translucent at 4ºC. The solution was changed once. The samples were placed in desiccator under vacuum (to help the solvents to penetrate into the samples) in open vials for 20 minutes at the beginning of the first change of each solution. After infiltration, the samples were embedded in the embedding solution. Sections 5 microns thick of the resin embedded material were cut with a dry glass knife in Leitz 1516 rotary microtome. The sections were collected with forceps and placed on a glass slide. The sections were then air dried. The air dried sections were stained with toluidine blue (0.5%) and heated slightly on a hot plate for few seconds. Then the sections were washed with distilled water and air dried once again. The sections were mounted and seen under a compound microscope. Various measurements were taken by an already standardized ocular micrometer and photographs were taken with the help of a microscope.

Results and Discussion

Preformed physical and chemical barriers are often considered as the first line of defense in plants against a pathogen before penetration (Cheng et al., 2012). In contrast to the earlier reports that stated that the resistant genotypes to leaf spot pathogen were characterized by lower stomatal index (Suryavanshi et al., 1994, Mayee and Suryavanshi 1995, Jyosthna et al., 2004), in the present studies it was observed that the mean stomatal number per microscopic field was more in case of the tolerant genotype as compared to the susceptible genotype (Table 1). The number of stomata between the abaxial surface and the adaxial surface and the length of stomata showed no significant differences in all the three genotypes. However, the tolerant genotype was characterized by a higher percentage of stomata and a wider stomatal pore diameter than the susceptible genotype (Table 2). The infection of biotrophic parasites, particularly leaf spot disease, required a state of maturity of the leaves and especially, the orifices of the stomata through which the penetration of conidia occurred. The diameter of leaf stomata orifices had to be greater than the germ tube of the pathogen, so that the penetration of the latter in leaf could take place (Tshilenge-Lukanda et al., 2012). Thus, from the present studies it was evident that not only the number of stomata, but the number of stomata with pore width of penetrable size, also acted as a factor in determining the tolerance to leaf spot pathogen. Smaller sized stomata were also reported in the groundnut cultivars tolerant to P. personata (Suryavanshi et al., 1994; Mayee and Suryavanshi, 1995; Jyosthna et al., 2004) and cocoa resistant to fungus, Oncobasidium theobromae (Anita-Sariand Susilo, 2013). In the present studies, the trichomes were present only on the abaxial surface and the tolerant genotype was characterized by the presence of more trichomes as compared to the susceptible genotype (Table 1). Increased number of hydrophobic pubescences (such as trichomes) may repel water from the leaf surfaces, change the leaf wettability and humidity in the canopy that interferes in spore germination, germ tube orientation, and stomata localization from getting into the plant tissue thus preventing successful penetration. Similar results were also reported by Chattopadhyay et al. (2011).

The total leaf thickness of the tolerant genotype was higher than the susceptible genotype. The thickness of palisade layer was more in case of the tolerant genotype (102.97 microns) as compared to the susceptible genotype (78.28 microns) (Table 3). Again, the palisade cell length was significantly higher in the tolerant and moderately susceptible genotype (49.53 and 50.72 microns) as compared to the susceptible genotype (42.97 microns). However, the breadths of the palisade cells were similar (Table 4). The palisade cells were densely packed, more layered with less intercellular spaces in the tolerant genotype than in the susceptible genotype. The spongy tissue thickness was not significantly different but the intercellular space in the spongy tissue of the susceptible genotype was more as compared to the spongy tissue of tolerant genotype (Fig 1a,b, & c). Higher palisade densities have been associated with resistance to several leaf spot pathogens (Mayee and Suryawanshi 1995, Yang, 2000, Smith et al., 2006). Evidence from the present studies suggested that compact palisade layers may slow or prevent the hyphal development due to the inability of the fungal pathogen to penetrate and colonise the tightly packed palisade cells. The loose arrangement of palisade cells, coupled with increased intercellular spaces in spongy parenchyma tissues in the susceptible genotype resulted in increased fungal ramification and progression in the leaves of the susceptible genotype. The quantitative measurements of spore attachment and germination indicated that these processes were similar in the examined genotypes and were not influenced by genetic differences of the hosts. The number of germ tubes per conidium was similar in all the three genotypes. Directed growth of germ tube towards stomata was more pronounced in the susceptible genotype. The percentage of penetration was significantly higher in susceptible genotype than in the moderately susceptible and tolerant genotype (Table 4). Fungal hyphae were less dichotomously branched and intracellular branched

haustoria formation was less in the tolerant genotype (Fig. 2a&b). Cell death was also evident in the tolerant genotype. Ramification of hyphae in the spongy tissue, stroma formation in the substomatal cavity, conidiophores formation and sporulation was highly reduced in the tolerant genotype (Fig. 2 c&d). The restricted development in the tolerant genotype resulted in an increased incubation period, latent period, and reduced lesion diameter (Table 5). Similar results were also reported by Muiru et al. (2008).

The present studies showed that the tolerant genotype was characterized by smaller size of stomatal pore, more trichomes, and high palisade index. The anatomical features of the leaves resulted in less ramification and sporulation of the fungal pathogen in the tolerant genotype. Thus, the studied micro morphological and anatomical traits are of potential value when selecting for late leaf spot resistance from progeny or from collected material used in groundnut breeding.

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Table 1. Average number of stomata, trichome per microscopic field and length of stomata in one month old leaves of selected groundnut genotypes.

	Number of Stomata per microscopic field		Length of Stomata (in µ)		Number of Trichomes on Abaxial Surface
Genotypes	Abaxial surface	Adaxial surface	Abaxial surface	Adaxial surface	
ICGV 86699 (tolerant)	34.13	31.13	20.20	17.79	8.02
ICGV 86590 (moderately susceptible)	26.52	27.90	20.25	16.63	5.18
TMV-2 (susceptible)	22.59	22.72	21.67	18.29	4.7
LSD at 1%	0.923		0.418		0.359
CV%	10.1		10.4		16.4

Table 2. Percentage distribution of stomata with stomatal pore size of 2.5, 3.75,and 5.0 micron (µ) in one month old leaves of the three selected groundnut genotypes.

Genotypes	Percentage distribution of stomatal pore size (in µ)					
	Adaxial surface			Abaxial surface		
	2.5	3.75	5.0	2.5	3.75	5.0
ICGV-86699 (tolerant)	66	33	1	68	31	1
ICGV-86590 (moderately susceptible)	45	51	4	54	43	3
TMV-2 (susceptible)	13	58	12	50	39	11

Table 3. Anatomical features of one month old leaves of selected groundnut genotypes.

Genotypes	Total leaf thickness (µ)	Palisade tissue thickness (µ)	Palisade cell length (µ)	Palisade cell breadth (µ)	Spongy tissue thickness (µ)
ICGV 86699 (tolerant)	249.06	102.97	49.53	13.96	56.17
ICGV 86590 (moderately susceptible)	264.78	108.89	50.72	14.15	52.28
TMV-2 (susceptible)	222.61	78.28	42.97	14.06	54.39
LSD at 1%	5.329	4.163	2.544	0.847	3.806
CV%	5.6	11.1	13.8	15.6	18.1

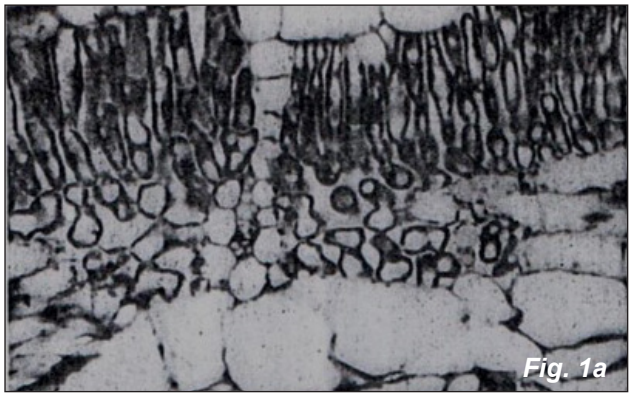
Table 4. Infection process of late leaf spot (LLS) pathogen on one month old leaves of the selected groundnut genotypes.

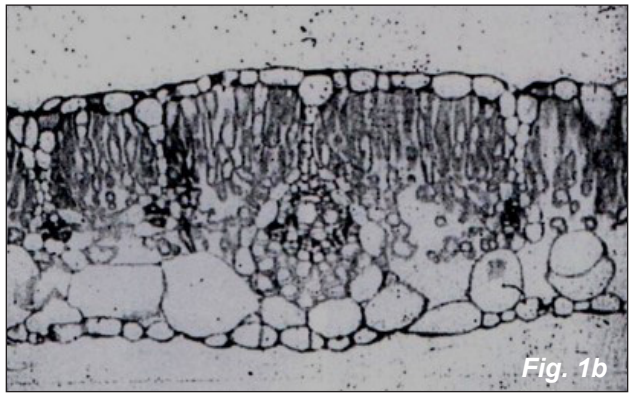
Genotypes	Percentage Germination		Percentage Penetration		
	12 HAI	24 HAI	72 HAI	96 HAI	114 HAI
ICGV 86699 (tolerant)	50	76	16.07	26	34
ICGV 86590 (moderately susceptible)	53	76	20	32.6	50
TMV-2 (susceptible)	60	75	38	54	64

Note. HAI -=Hours After Inoculation

Table 5. Time course of the late leaf spot (LLS) disease development after inoculation with LLS pathogen in the selected groundnut genotypes.

Groundnut genotypes	Incubation period (days)	Latent period (days)	Lesion diameter (mm)
ICGV-86699 (tolerant)	12	25	3
ICGV-86590 (moderately susceptible)	9	15	2.4-5.4
TMV-2 (susceptible)	7	12	2.8- 6.4





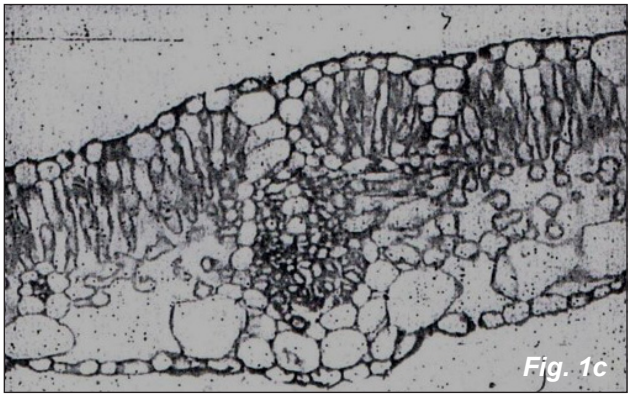
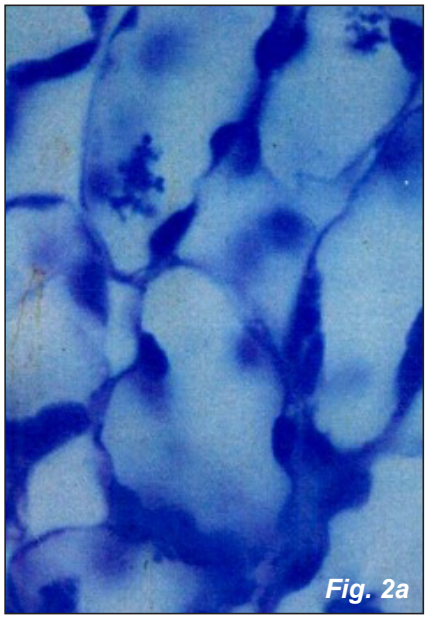
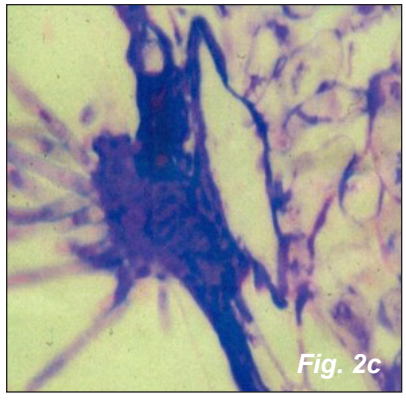


Fig.1: Light micrographs of 5 µ thick microtome leaf sections of the three selected groundnut genotypes showing anatomical differences (a) tolerant genotype-ICGV 86699 (b) moderately susceptible genotype-ICGV 86590 (c) susceptible genotype-TMV 2





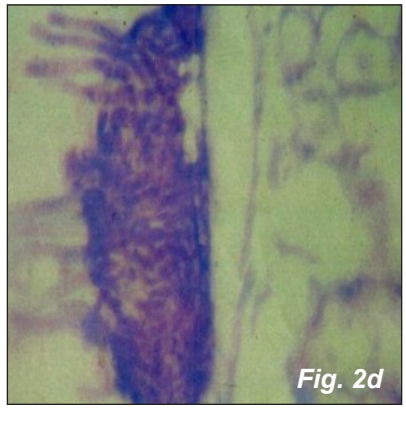


Fig. 2: Light micrographs of groundnut leaf infected with LLS pathogen showing restricted development of intracellular haustoria and sporulation in the tolerant genotype-ICGV 86699 (a, c) as compared to susceptible genotype-TMV 2 (b, d).