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IDENTIFICATION OF SOURCES OF RESISTANCE TO GREY MILDEW DISEASE (*RAMULARIA AREOLA*) IN DIPLOID COTTON (*GOSSYPIUM ARBOREUM*)

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FOREWORD

Cotton, a principal cash crop touches India's economy at several points. An estimated 60 million people are dependent for their livelihood, either by its cultivation, marketing, processing, export, textile manufacturing or by extraction of seed oil and production of 'Vanaspati'. In recent years with increasing International demand and domestic consumption, the cotton productivity, fibre quality improvement as well as resistance breeding against insect pests and diseases has become more mission oriented. However, better management of insect pests and diseases need to be considered as a part of built-in mechanism of crop production system. The advent of transgenic cotton and its cultivation in India (intra-*hirsutum* hybrids/varieties) should be considered as a beginning of new era in cotton cultivation of the country. Internationally, genetic engineering and molecular level work has paved the ways to develop transgenic varieties resistant to insect pest (bollworms) and diseases (Fusarium wilt, Verticillium wilt). Development of insect pests and disease resistant varieties ultimately benefits the farmers and country to a large extent by reducing the cost of cultivation and also by allowing lesser consumption of harmful agricultural chemicals. This in turn helps in avoiding environmental pollution and the mammalian toxicity. Incorporation of resistance in cultivated varieties however can be possible, only with maintenance and preservation of valuable germplasm and the wider range of genetic variability. Identification of sources of resistance against economically important diseases can help in developing resistance breeding programmes. The grey mildew disease (*Ramularia areola*) affecting cotton foliage has been, and still continues to be a very important disease in India since last several decades. Scientists at CICR, Nagpur during last 20 years have made it possible to achieve a milestone with identification of sources of resistance. Similar attempts are on way at CICR, Nagpur and its Regional stations at Sirsa (Haryana) and Coimbatore (Tamil Nadu) which I hope will help in tackling other economically important foliar diseases namely, the leaf curl virus disease and bacterial angular leaf spot.

I am sure that the bulletin on "Identification of sources of resistance to grey mildew disease (*Ramularia areola*) in diploid cotton (*Gossypium arboreum*)" will be useful for furthering the cause of diseases management in cotton.

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Director

IDENTIFICATION OF SOURCES OF RESISTANCE TO GREY MILDEW DISEASE (*Ramularia areola*) IN DIPLOID COTTON (*Gossypium arboreum*)

Introduction

From time immemorial, India was the only country known for its cotton fabrics, the rest of the world being clad mostly in wool flax and silk. The Babylonian and Greek names Sindhu and Sindon respectively for cotton clearly point out that Indus valley civilization (3000 BC) was the home of cotton growing. Marco Polo in a detailed account of his visit to India during the 13th century AD mentioned that Masuli pattam produced the finest and the most beautiful cottons to be found in any part of the World. This was particularly from Asiatic *Gossypium arboreum* and *Gossypium herbaceum*, which were indigenous to India. From 1500 BC to 1700 AD India was recognized as the cradle of cotton industry. Over 3000 year monopoly in cotton muslins ended in less than 30 years of the onset of Industrial revolution in England and development of Lancashire cotton industry. Archaeological excavation of about 3000 BC in Indus River Valley revealed the use of cotton fibre. Cotton enjoyed a pride of place among cash crops from the earliest times. Cotton finds mention in the *Rig Veda* and *Manu's Dharma Shashtra* (Narayanan *et.al*.2002).

India is the original home of domestication, diversification and development of the Asiatic cultivated cottons representing *Gossypium herbaceum L.* and *Gossypium arboreum L.* Until almost upto independence of India, the country was growing the two Asiatic cottons which occupied area of the total 97 % area under cotton. However, since independence the acreage under *hirsutum* cotton has phenomenally increased to occupy 72 per cent area of the total area of nine million hectares. The acreage under Asiatic diploid cotton area has declined from over 97 per cent a few years before independence to around 28 per cent at present.

Inherently, low yielding nature and the low quality of diploid cottons with short to medium staple quality compared to the American cottons have caused rapid downfall of diploid cotton in the second half of the century. Researches to improve the yield potential and fibre quality of the two Asiatic cotton species commenced almost from 1901 and continued vigorously upto 1960. However, in the meantime while American upland cotton (*G.hirsutum*) and its hybrids received more emphasis and diploid cotton research received some setback. Because of high economic returns, American cotton could spread faster and eventually replaced diploid cottons to a larger extent.

The indigenous *Gossypium arboreum* cotton is mainly cultivated in India, Pakistan, Bangladesh, Sri Lanka, Myanmar, China and Vietnam, and contributes around 2 to 3 per cent of the total global production. In India, *arboreum* cotton is cultivated in all the nine major cotton growing states of the country i.e. Punjab, Haryana, Rajasthan, Madhya Pradesh, Maharashtra, Gujarat, Andhra Pradesh, Karnataka and Tamil Nadu. Besides these states, it is also cultivated in Assam, Tripura, Nagaland in North Eastern Hill Region on small area.

In India diploid cotton covers 28 per cent area. *Arboreum* cottons cover about 17 per cent of area and contributes around 12 per cent of the total national production.

Geographical range of distribution and cultivation of races of *Arboreum* cotton in India:

Name of State	Race (s) grown
Punjab	*Bengalense*
Haryana	*Bengalense*
Rajasthan	*Bengalense*
Madhya Pradesh	*Bengalense*
Maharashtra	*Bengalense*
Gujarat	*Bengalense*
Andhra Pradesh	*Bengalense, Indicum*
Karnataka	*Bengalense*
Tamil Nadu	*Indicum*
Assam	*Cernuum*
Meghalaya	*Cernuum*

Main features of arboreum cotton

1. *Arboreum* cottons have wide adaptability and therefore can be cultivated under harsh climatic conditions.
2. They are more tolerant to salinity and moisture stress due to lesser leaf area and deeper root systems.
3. *Arboreum* cottons have high degree of tolerance to sucking insect pests such as jassids, aphid and thrips.
4. *Arboreum* cottons show more endurance consistency toward fibre properties year after year.
5. The short and coarse fibre of *arboreum* cottons have high moisture absorbancy and luster.
6. Lint of some varieties of *arboreum* cotton is highly suitable for chemical treatment. Such varieties are called as easy care cottons. The fabrics made from such cotton are easily washable and need less maintenance.

Arboreum cotton possesses several above useful characters. However, there are some drawbacks in *arboreum* cotton such as tall growing plant habit, less number of flower flushes, no rejuvenation plant capacity, short and coarse fibre, poor yield, small size of bolls, hanging bolls, high locule shedding and brittle pedicel.

Concerted efforts were made for genetic improvement in diploid cottons especially in *G. arboreum* and the remarkable progress has been made in genetic improvement of this species during last three decades. (Singh and Punit Mohan *et. al.*, 2002).

Character	Present Status	Improvement
Fibre length (mm)	25.1	29.4
Fibre strength (g/tex)	17.7	23.0
Ginning Outturn Percentage (%)	37.0	42.0
Boll weight (g)	2.0	3.5

Earliness (days)	170	155
Dwarf type (cm)	200	60 to 80
Locule retentivity (days)	5	20

History of grey mildew disease:

The grey mildew disease (*Ramularia areola* Atk.) was reported for the first time on upland cotton (*Gossypium hirsutum* L.) in Auburn, Alabama, USA in the year 1890 and was recorded as 'Areolate mildew' of cotton (Atkinson, 1890). Subsequently, the disease was reported by many workers in cotton-growing areas of the world, affecting all the four commercially cultivated *Gossypium* species i.e. *Gossypium arboreum*, *G.herbaceum*, *G.hirsutum* and *G.barbadense* (Bell, 1981a). The disease was recorded under various symptom descriptive names such as areolate mildew, false mildew, frosty blight, white mold and grey mildew. In Maharashtra State, the grey mildew is commonly referred to as 'Dahiya' or 'Dahya' disease because of the symptoms resembling sprinkled curd on foliage (Gokhale and Moghe, 1965). In India, the disease has been reported to occur in Tamil Nadu, Andhra Pradesh, Karnataka, Bihar, Madhya Pradesh, Gujarat, Maharashtra (Butler, 1918; Uppal, 1948; Reddi and Ranganadhacharyulu, 1960; Vasudeva, 1960, 1962; Gokhale and Moghe, 1965), Punjab (Chopra *et. al*., 1980; Sharma *et. al.,* 1986) and Haryana (Chauhan, 1985), affecting very severely the diploid Desi/ Asiatic G. *arboreum* and G. *herbaceum* cottons.

Diseases of 'Diploid' cotton (Gossypium arboreum, G.herbaceum) in India:

The important diseases affecting 'Diploid' cotton in India include Alterneria leaf spot (*Alternaria macrospora*), grey mildew (*Ramularia areola*), Fusarium wilt (*Fusarium oxysporum* f. sp. *vasinfectum*), anthracnose (*Colletotrichum indicum Dastur)* and Rhizoctonia root rot / Macrophomina stem break (sclerotial stage *Rhizoctonia bataticola*, pycnidial stage *Macrophomina phaseolina*).

Estimation of yield losses and Economic significance:

Systematic studies on the effect of grey mildew disease on *G.arboreum* cultivars (AKA 235, G 1946, Y-l) on account of yield losses in Vidarbha region of Maharashtra State has been carried out wherein seed cotton yield losses ranging between 21to 26 per cent has been reported (Kodmelwar,1976).

In India, seed-cotton yield losses ranging between 26 to 66 per cent have been reported under grey mildew epiphytotic conditions in the States of Tamil Nadu, Andhra Pradesh and Maharashtra. Under exclusive monoculture of 'Diploid' cotton, the losses extending even upto 90 percent has been recorded (Sangitrao *et. al.,* 1993). In intra-*hirsutum* hybrid H4 (tetraploid cotton), the loss in yield to an extent of 62 to 68 per cent, has been reported in chemically unprotected crop, in disease endemic area of Akola district of Maharashtra (Shivankar and Wangikar, 1992).

Because of the great economic significance of the disease, detailed investigation on

physiology of the fungus and its infectivity pattern (Chidambaram and Johnson, 2002;

Vamadevaiah *et. al.,* 2004), role of perithecia (ascospores formation) in perpetuation of the fungus (Chattannavar *et. al.,* 2001) as well as studies on molecular markers in resistance breeding programme (Amudha *et. al.,* 2004) has been taken up in India, in recent years.

Grey mildew epidemics and disease severity:

A descriptive account of the grey mildew disease and its causal fungus *Ramularia areola* (Synonyn - *Ramularia gossypii*) has been given earlier in books on cotton diseases (Butler, 1918; Uppal, 1948; Vasudeva, 1960, 1962; Hillocks, 1992; Srinivasan, 1994), compendiums (Watkins, 1981; Sangitrao *et. al.,* 1993). Exclusive studies on pathogen taxonomy, physiology and nomenclature have also been published earlier in the form of research papers and descriptions (Ehrlich and Wolf, 1932; Gokhale and Moghe, 1967a, 1967b; Mulder and Holliday, 1976; Rathaiah, 1976).

In Central India, the occurrence of grey mildew disease has been reported in destructive epidemic form during the years 1958, 1988 and 1993 in the month of October-November in June-July sown rainfed crop. Occurrence of grey mildew epidemics and records of severe outbreaks in different parts of India has been given in Table 1.

Table 1: Grey mildew epidemics and severe outbreak records in India

Sr.No.	Place/Province	Crop season years of epidemic / Severe out-break	Authors/Source
1	Kovilpatti, South Arcot district (Tamil Nadu)	1929-30 and 1930-31	Anonymous (1931)
2	Hagari (Karnataka)	1932-33 and 1933-34	Anonymous (1934)
3	Kovilpatti, South Arcot district (Tamil Nadu)	1941-42	Thomes (1942)
4	Nandyal (Andhra Pradesh)	1948-49	Anonymous (1949)
5	Baptla (Andhra Pradesh)	1953-54	Govindarao and Subbaiah (1954)
6	Vidarbha Region (Maharashtra)	1958-59	Siddiqui and Sahni (1962)
7	Vidarbha Region (Maharashtra)	1962-63	Gokhale and Moghe (1965)
8	Amravati district, Vidarbha Region (Maharashtra)	1964-65	Gokhale and Moghe (1967 a)
9	Nagpur district, Vidarbha Region (Maharashtra)	1967-68	Gokhale and Moghe (1967,a)
10	Marathwada Region (Maharashtra)	1968-69	Kolte (1973)
11	Akola and Amravati districts, Vidarbha Region (Maharashtra)	1977-78	Holey and Moghe (1977)
12	Ranchi (Bihar)	1979-80	Dutta and Jha (1979)

13	Kozhipakkam, South Arcot district (Tamil Nadu)	1979-80	Dake and Kannan, (1982)
14	Nagpur district, Vidarbha Region (Maharashtra)	1980-81	Holey and Somani (1980)
15	Marathwada Region (Maharashtra)	1980-81	Badgire (1980)
16	Kovilpatti, South Arcot district (Tamil Nadu)	1980-81	Dake and Kannan (1981)
17	Vidarbha Region (Maharashtra)	1988-89	Mukewar (1989)
18	Vidarbha Region (Maharashtra) and Saunsar Region, Chhindwara (Madhya Pradesh)	1993-94	Mukewar *et. al.* (1994)

Screening of 'Diploid' cotton (G.arboreum) germplasm against grey mildew:

I. Germplasm screening:

The screening programme for resistance to grey mildew disease was undertaken at CICR, Nagpur during the years 1985 to 1989. A total number of 1489 G. *arboreum* germplasm lines were evaluated to the disease by artificial inoculations. In field screening, among the lines evaluated, a total number of 24 lines were observed free from infection which were inoculated further in pot culture during the years 1989 to 1992. The final screening and evaluation of germplasm, revealed the presence of 7 immune lines (No infection) and 17 highly Resistant lines (Mukewar *et. al.*, 1995). These 24 lines have been incorporated into resistance breeding programme at CICR, Nagpur.

II. Preparation of inoculum and artificial inoculation for germplasm screening:

Artificial inoculation of *G.arboreum* test germplasm lines was carried-out in the field as well as in pot culture in screen house. The experimental sowing of test germplasm lines was undertaken with the onset of monsoon either in the last week of June or first week of July. In screening of *G. arboreum* test material in the field, it was surrounded by five infector rows each of grey mildew highly susceptible *G. arboreum* cultivar AKH 4 and *G. herbaceum* cultivar Jayadhar. The *G. herbaceum* cultivars (Not cultivated in Vidarbha region of Maharashtra) which are usually of long duration with dark green foliage, has been used as infector rows to provide conidial inoculum for repeated cycles of infection for longer period. The artificial inoculation of *G. arboreum* germplasm lines and that of infector rows was carried-out in 30 to 40 days old crop. The intermittent rains with humid atmosphere allowed spread of the disease faster in the field. The infector rows of cultivars AKH 4 and Jayadhar served as permanent source of inoculum in the field during the crop season for screening of *G. arboreum* test material. For artificial inoculations in the field and screen house, conidial suspension was prepared in tap water. Two to three inoculations were made on 30 to 40 days old plants usually during the cloudy weather so as to obtain desired germination of the conidia of grey mildew fungus and its establishment for the purpose of infection. In the preparation of inoculum, AKH 4 and Jayadhar grey mildew infected leaves were collected from ratoon plants. The ratoon plants of these cultivars were maintained in pots in the screen house where upon the infection appeared every year with the onset of

monsoon. The white, powdery growth of the fungus *R. areola* appeared prominently in the month of August coinciding the cool, cloudy, rainy weather. In the preparation of conidial/spore suspension in water, the diseased leaves from ratoon plants were collected in the morning hours. The leaves were then soaked in tap water (50 leaves/l000ml water) in the plastic tub for 7-8 hrs. and subsequently rubbed with plastic tea strainer so as to remove apparently visible fungus growth on upper and under surface of the leaves. The conidial suspension was finally sieved through plastic strainer/muslin cloth for removal of foliage pieces. The white turbid conidial suspension in water was further diluted to obtain 36000 conidia / ml of water. The conidial inoculum thus obtained was then sprayed with 'Aspee' baby sprayer in the evening hours in the field and in screen house. The inoculum spraying was always carried-out in the evening hours so as to provide cool, humid atmospheric conditions, which are congenial for germination of conidia in a film of water on foliage.

Disease symptoms:

In grey mildew infection, both surfaces of the leaves get uniformly covered by white powdery growth of the fungus (Photo plate No.l (a)). This further leads to curling and drying of the leaves (Photo plate No. l (b)) and the diseased leaves defoliate eventually. Initial infection appears as triangular, square or irregularly circular whitish spots of 3 to 4 mm size on leaves. As the disease severity increases, the smaller spots merge together and form bigger spots. Whitish powdery growth of the fungus appears first on under surface of the leaves and visible later on the upper surface. The whitish powdery growth visible on under surface initially, has reversely a yellowish colour spots on upper surface of the leaf. As the disease develops, appearance of white frosty growth of the fungus becomes more apparent. Due to intense infection, shedding of leaves takes place and only the naked branches remain in the field. Appearance of white / grey spots because of the raised aerial growth of the fungus resemble sprinkled curd or 'Dahi' and hence the disease is referred to as vernacularly 'Dahiya' or 'Dahya'. Under prolonged favourable atmospheric conditions, disease aggravates and the mildew spots appear on bracts and bolls. Shedding of leaves and buds takes place and further development of the newly formed bolls is affected. In India, under exclusive monoculture of 'Diploid' cotton (*G. arboreum*) prior to the year 1970, grey mildew used to appear late in crop season resulting in defoliation, which in turn facilitated easy hand-picking of seed cotton and hence the disease was not attended to very seriously.

During the year 1993, the disease appeared in epidemic form in Central India. Surprisingly, the occurrence of grey mildew was noticed on one month old plants of *G. arboreum* cultivar AKH 4 (Photo plate No.1 (c & d), causing alarming concern for cotton cultivation.

Disease Rating and fibre properties evaluation of test germplasm:

Artificial inoculation of test *G.arboreum* germplasm lines was undertaken in the field and in pot culture simultaneously. Disease intensity was recorded 15 and 30 days after the inoculation, using infection rating chart developed by Kodmelwar (1972) for *G. arboreum* varieties. Observations on disease severity and the disease incidence were recorded on two

leaves from upper, middle and lower portion of each plant. The average disease intensity on the leaves and foliar incidence of the disease were calculated according to the formulae developed by Lanjewar *et al.* (1975) as below:-

$$\text{Disease severity percentage} = \frac{\text{Total percentage disease intensity on leaves under observation}}{\text{Total number of leaves under observation}} \times 100$$

$$\text{Foliar incidence of disease percentage} = \frac{\text{Number of leaves affected}}{\text{Total number of leaves}} \times$$

The germplasm screening against grey mildew of *G.arboreum* lines was grouped as: Immune (No disease), Highly Resistant (rating index 0.1-5.0%), Resistant (5.1-10.0) and Susceptible (10.1-40.0). A total number of 1489 *G. arboreum* germplasm lines were evaluated for grey mildew reaction at CICR, Nagpur wherein 7 lines were observed Immune (No disease symptoms) which are namely, Bangladesh (EC 174092), G-135-49, 30805, 30814,30826,30838 and 30856 while, 17 lines showed Highly Resistant reaction which are namely, AC 24, AC 631, AC 655, AC 727 DH, AC 727 MH, Desi 1, G-1l2, Gao-CB-3 NLL,H-574, K 53-519, Malvi 20,30802,30815,30821,30843,30845 BLL and 30845 MLL.

The lint samples of 7 Immune lines (No disease) and 17 Highly Resistant lines were analysed for staple length, fibre fineness and other attributes at Central Institute for Research on Cotton Technology (CIRCOT, Mumbai), substation at Nagpur (Punit Mohan *et. al.*, 2001.). The disease index and economical characters are presented in Table 2.

Table 2: Field and Screen-house rating of G. arboreum germplasm lines immune and resistant to grey mildew.

Germplasm Accession	Disease index (%) during			Fibre property			
	1989- 90	1990- 91	1991- 92	2.5 % span length (mm)	Uniformity ratio (%)	Fineness micronaire 10^{-6} g/m	Bundle strength Tenacity (g/t 3.2mm)
Bangladesh (EC 174092)*	--	--	--	18.4	49	7.4	15.3
G 135-49*	--	--	--	21.8	50	7.5	19.7
30805**	--	--	--	20.2	50	7.9	17.9
30814 **	--	--	--	17.5	52	Above scale	16.4
30826**	--	--	--	18.5	49	Above scale	17.0
30838**	--	--	--	20.3	50	Above scale	16.6

30856**	--	--	--	17.0	48	Above scale	16.0
AC 24*	3.6	3.5	3.4	24.7	49	4.3	20.2
AC 631*	4.2	4.0	3.9	24.3	50	5.2	20.4
AC 655*	3.4	3.2	3.2	24.4	50	5.2	22.3
AC 727 DH*	4.4	4.1	4.0	23.4	50	4.8	21.2
AC 727 MH*	4.4	3.9	4.0	23.0	48	4.6	17.1
Desi 1*	4.3	3.8	3.7	23.8	50	4.6	20.5
G 112*	4.2	3.6	3.6	22.7	51	5.7	17.4
Gao-CB-3 NLL*	2.8	2.5	2.6	24.8	47	5.1	21.2
H 574*	4.6	3.8	3.9	25.9	52	4.8	25.4
K53-519 *	4.8	4.2	4.1	24.8	49	5.6	20.8
Malvi20 *	3.6	3.4	3.5	24.9	48	5.3	22.8
30802 **	2.8	2.6	2.6	23.1	49	5.4	18.0
30815 **	2.8	2.6	2.5	17.9	48	7.0	14.5
30821**	3.4	3.2	3.3	22.3	51	5.1	19.8
30843 **	3.8	3.6	3.5	24.1	48	4.8	20.0
30845 BLL **	3.8	3.6	3.5	24.8	45	4.6	17.5
30845 MLL **	3.8	3.6	3.6	25.5	49	4.2	20.3
AKH4 (control)	38	34	32	24.9	50	4.9	19.5

**Gossypium arboreum Race bengalense, ** Gossypium arboreum Race cernuum*

Grey mildew epidemiology and weather factors influencing disease outbreak:

Long spell of moist humid weather has been reported to favour the development and spread of grey mildew disease. The disease has been recorded to cause severe damage under weather conditions of above normal, well distributed and intermittent rains. There are records of complete destruction of the cotton crop in wet-humid crop seasons in Central region of the India as well as in the States of Tamil Nadu, Karnataka and Andhra Pradesh. Occurrence of above average rainfall in the months of September and October has been recorded to increase disease severity in Central India.

Epidemiological studies carried out in the disease endemic area of Akola district of Maharashtra State by Shivankar (1989), showed that the heavy rainfall (833.2 mm) during June to September, minimum and maximum temperature in the ranges of 19.7-23.7 and 29.4-30.9^{0}C, respectively and relative humidity (RH) between 78-85% in the morning hours and 45.5-62% in the evening hours, contributes for grey mildew development.

In another studies on factor analysis in occurrence of grey mildew epidemics in Central India during the years 1988 (Mukewar, 1989) and 1993 (Mukewar *et. al.*, 1994), it was observed that the minimum and maximum temperature in the ranges of 24-25^{0}C and 28-31^{0}C respectively, relative humidity (RH) between 90-91 % and cultivation of highly susceptible *G.arboreum* cultivar AKH 4 were responsible for grey mildew epidemics.

Biochemical basis of disease resistance in Immune and Highly Resistant lines:

Considering the records of grey mildew epidemics in Central and South India, several sources of resistance especially in diploid cottons (*G. arboreum, G. herbaceum*) have been identified in the past however, the biochemical basis of disease resistance was not worked-out in detail. With the identification of immune and highly resistant germplasm lines in 'Diploid' cotton (Mukewar *et. al.,* 1995), studies were undertaken at CICR, Nagpur to understand the role of biochemical factors associated with resistance to grey mildew. The test plants grown in pot culture in glasshouse, comprised of the immune *G. arboreum* germplasm line G-135-49, highly resistant lines Desi-l, AC 24 and 30821 and, highly susceptible cultivars AKH 4 (*G. arboreum*) and Jayadhar (*G. herbaceum*). These were analysed for biochemical studies on pathogenic intection (Chakrabarty *et. al.,* 2002).

In biochemical studies at CICR, the Phenylealanine ammonia lyase (PAL) enzyme activity was assayed in healthy and diseased leaves. Healthy leaves of grey mildew resistant lines exhibited less PAL activity than that of grey mildew susceptible cultivars AKH-4 and Jayadhar. On the other hand, cultivars AKH-4 and Jayadhar possessed higher level of constitutive phenol, gossypol and flavonol in comparison to grey mildew highly resistant germplasm lines. Contrary to analysis of plants grown in glasshouse, the field grown immune line G-135-49 exhibited highest PAL activity as well as the levels of total phenol, gossypol and flavonol. The induction of PAL activity in field grown immune line G-135-49 appeared to be due to the presence of fungus propagules (*R.areola*) naturally/artificially available in the atmosphere and their attempt to establish in host system. This type of phenomenon with the activation of defence system has been reported earlier in resistant cotton line in studies conducted in USA (Bell, 1981a). In present studies, the PAL enzyme was observed as the key enzyme for synthesis of the phenolics in plants, which usually contributes for forming defensive mechanism in host plant against the pathogenic micro organisms attack.

Review of literature indicates the chemical gossypol to be associated with protection in cotton against boll afflicted maladies. In a countrywide survey conducted in China for recording the incidence of cotton boll rot complex due to several pathogens, it was found that the cultivars of low gossypol content had higher boll rot incidence.Gopalakrishnan *et. al.,* (1992) reported 20-30 per cent reduction in gossypol and tannin contents of the cotton leaves affected due to grey mildew and bacterial blight. The ability of resistant plants to elicit greater induction of defense related PAL activity and phenolic compounds in response to infection, appears to be responsible for less damage.

It was observed in the studies at CICR, that the resistant plants possessed higher constitutive tannin compared to susceptible ones. Tannin was further induced over their constitutive levels in response to infection. The magnitude of induction was higher in grey mildew resistant germplasm lines in comparison to susceptible cultivars. The immune line G-135-49 when grown under field condition, showed 47 per cent higher tannin content compared to

same line grown under the glasshouse condition. The healthy plants of resistant germplasm lines and susceptible cultivars possessed significantly different levels of amino acid. No specific trend or correlation could be observed for amino acid content with reference to inherent resistance or susceptibility to grey mildew. Highly resistant *G. arboreum* germplasm line AC 24 possessed highest concentration of constitutive amino acid. Nevertheless, the susceptible cultivar AKH 4 exhibited higher amount of amino acid, compared to highly resistant lines Desi 1 and 30821 including the immune line G 135-49. The amino acid content of immune line G-135-49 was minimum. Grey mildew highly resistant lines possessed significantly higher constitutive proline in comparison to susceptible plants. Proline concentration declined with the development of disease, with minimum level in moderately infected plants. The immune line G-135-49 possessed highest concentration of proline when grown both under glasshouse and field condition. Healthy leaves of highly susceptible cultivars AKH 4 and Jayadhar possessed significantly higher amounts of total sugar than that of resistant and immune lines. As a result of infection, the concentration of total sugar declined more rapidly in susceptible lines in comparison to resistant ones (Chakrabarty *et. al.*, 2002). Peroxidase isozyme pattern of different groups of *Ramularia areola* has also been studied at Dharwad (Vamadevaiah *et. al.*, 2004).

Biochemical studies at CICR, Nagpur indicate that no correlation appears to exist between the constitutive levels of PAL, total phenol, gossypol, flavonol and inherent resistance of the plants against grey mildew. With regard to biochemical factors governing resistance in diploid cotton (*G. arboreum, G. herbaceum*) against grey mildew (*Ramularia areola*), it was observed that the induced rather than the constitutive levels of phenylalanine ammonia lyase (PAL), total phenol, gossypol and flavonol play crucial role in governing resistance. Studies at CICR lead to conclusion that besides biochemical factors, the inherent resistance in cotton against grey mildew appears to be dependent upon several complex and inter-related factors viz, histo-chemical, morphological and anatomical.

Life cycle of the pathogen:

The asexual form of the grey mildew fungus i.e. the imperfect conidial stage is known as *Ramularia areola* Atk. (synonyms=*Ramularia gossypii* (Speg.) Ciferri, *Cercosporella gossypii* Speg.), while the Ascomycetous sexual stage i.e. the perfect state of the fungus is known as *Mycosphaerella areola* Ehrlich and Wolf. The conidia (asexual spores) are hyaline (colourless), straight, cylindrical, usually pointed at both ends or occasionally rounded, 1-3 septate, mostly 2 septate. The conidia are formed in chain (catenulate) and are branched some times. The size of the conidia measures 14 to 37 mm in length and 2.5 to 5 mm in breadth (1mm =1/1000 of mm). The grey/white aerial growth of the fungus visible in diseased spots if examined under compound microscope in a drop of water, reveals cylindrical colourless conidia. The conidia/asexual spores cause infection after the germination in a film of water on leaves and cause secondary spread of the disease in the field. The aggravation of the disease is mainly dependant on high atmospheric humidity (90-91 % RH), cool temperature (23-27°C) and susceptible cotton varieties.

On susceptible cultivars belonging to *G. arboreum*, the conidial multiplication is faster with repeated conidial cycles leading to disease epidemics. Cotton being a long duration crop of 150 to 180 days maturity harvest, the cool, wet-weather plays a vital role in disease outbreak.

Carry over of the fungus from season to season and Primary infection:

The conidial stage / asexual spore form (Imperfect state) of the grey mildew fungus in known as *Ramularia areola*, while the Ascomycetous stage/sexual spore form (Perfect state) is known as *Mycosphaerella areola.* The grey mildew fungus develops into three distinct stages during its life cycle viz., i) formation of conidial stage on green leaves during the crop season which is adapted for secondary spread of the disease followed by ii) development of a spermogonial stage (male-female meeting) in diseased defoliated leaves fallen on ground, and further iii) the development of sexual ascigerous stage in decaying/ withering leaves with formation of ascospores in perithecial bodies (Ehrlich and Wolf, 1932; Gokhale and Moghe, 1967a, b). The ascospores have been recorded to serve as the primary source of inoculum for next season crop. With the onset of monsoon, the ascospores shoot-out from perithecial bodies and cause initial infection on young seedlings. The fungus grows further and form conidia, which are responsible for secondary spread of the disease in the field.

Aerobiological studies in the spread of grey mildew conidia:

Aerobiological studies were undertaken for observing the aerial dissemination of *Ramularia areola* conidia during the crop season of 1984-85 and 1985-86 at Nagarjun Sagar, Andhra Pradesh (Raghuram and Mallaiah, 1990). Air sampling for *R. areola* conidia was carried out in cotton fields with cultivar MCU-5 (*G. hirsutum*), using the 'Burkard spore trap'. The air-borne conidia of grey mildew fungus were trapped during the months of November to January in July/August sown crop ageing between 116 to 123 days. The highest daily mean of conidia numbering between 11 to 28/m^3 were observed in the month of November. The air-borne conidia of *R. areola* showed afternoon pattern of circadian periodicity with peak occurring at 16.00h. The conidia were observed in the air when the temperatures ranged between 27-31^{0}C (maximum) and 18-23^{0}C (minimum), and the percentage relative humidity (RH) between 70-90 at 07.0 h and 55-75 at 14.00 h. The hyaline (colourless), single celled to 1-3 septate, oblong conidia with pointed, rounded or flattened ends measuring 10-35 X 4-5 mm were observed on spore trap slides.

Anatomical studies of *Gossypium arboreum* lines Immune to Grey Mildew Disease

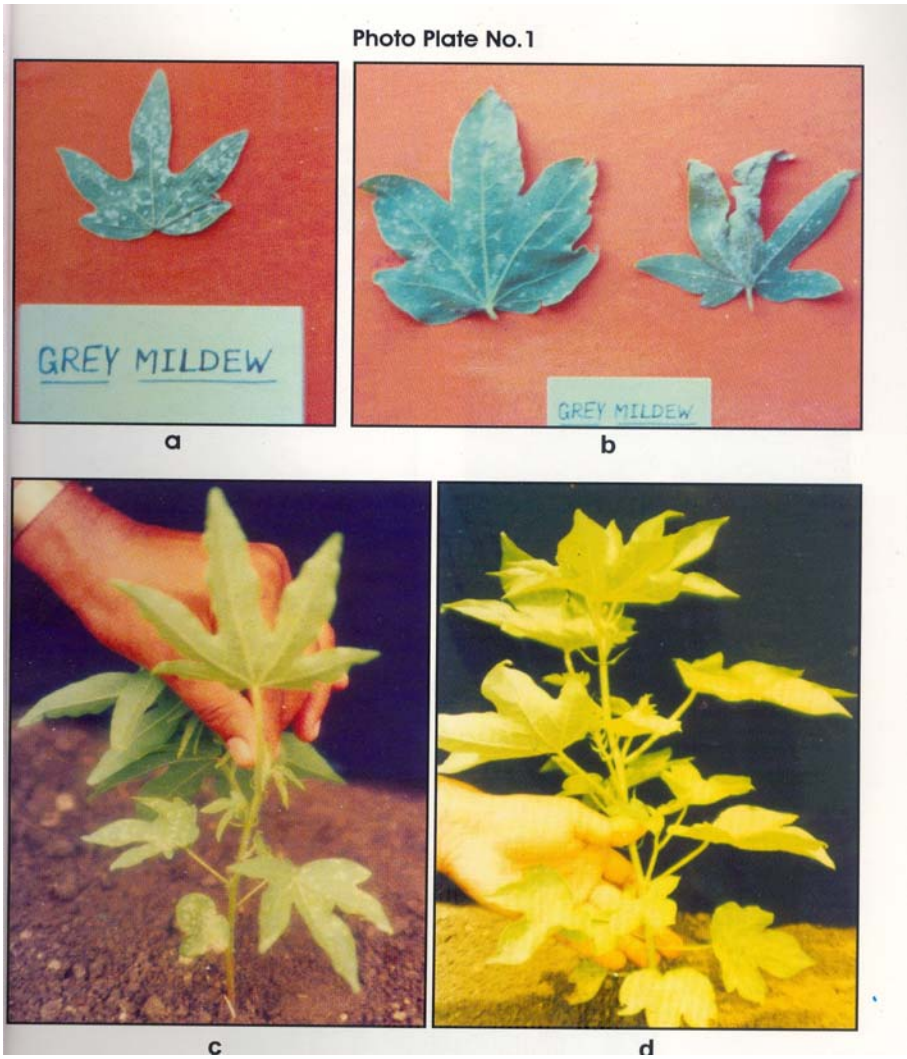
Seven immune *G.arboreum* germplasm lines namely, 'Bangladesh (ECI74092) and G. 135-49 (Photo plate No 2 (a)) collected from the Bangladesh and Punjab State of India, respectively and other five lines namely, '30805' (Photo plate No 2 (b)) '30814' (Photo plate No 3 (a)) 30826' '30838' (Photo plate No 2 (c)) and '30856' (Photo plate No 3 (b)) collected from North-Eastern Region of India were used in the investigation. The susceptible *G. arboreum* cultivars 'AKH-4' and 'G 27' served as the control.

High degree of variability on anatomical features among the immune and susceptible plants to grey mildew pathogen was observed. The cuticle thickness among immune lines was in the range of 3.690 to 5.166 µ in comparison to 2.460 µ observed in both the susceptible cultivars 'AKH 4' and 'G 27'. Similarly, the thickness of lamina ranged between 110.70 to 115.62µ in immune lines as compared to 108.24µ in 'AKH 4' and 105.75 µ in 'G 27'. Higher thickness of leaf cuticle and the lamina in immune lines indicated positive correlation when compared with standard suscepts. Among immune lines, the accession 'Bangladesh' (EC174092) showed highest values for cuticle and lamina thickness character (Punit Mohan *et. al.,* 1997). Details of the anatomical studies conducted at CICR, Nagpur are given in Table 3.

Table 3: Leaf anatomical features of immune and susceptible *Gossypium arboreum* germplasm lines to grey mildew (*Ramularia areola*).

Germplasm accession	Cuticle thickness (µ)	Lamina thickness (µ)	Thickness covered by palisade parenchyma (µ)	Thickness covered by spongy parenchyma (µ)	No. of epidermal cells	No. of stomata
Bangladesh (EC174092)	5.166	115.62	46.90	39.52	19.31	71.19
G 135 -49	4.936	113.16	51.82	39.52	19.23	67.50
30805	4.166	113.16	41.98	39.52	19.45	69.31
30814	4.920	113.16	41.98	39.52	19.38	69.28
30826	4.920	113.16	41.98	39.52	19.55	71.53
30838	4.920	110.70	41.98	39.52	21.74	71.46
30856	3.690	113.16	41.98	39.52	19.55	71.81
AKH 4	2.460	108.24	37.06	39.52	14.77	89.44
G 27	2.460	105.75	37.48	39.52	17.53	103.35
Range (immune)	3.690 5.166	110.70 115.62	41.98 51.82	39.52 39.52	19.23 21.74	67.50 71.81
Range (susceptible)	2.460 - 2.460	105.75 - 108.24	37.06 - 37.48	39.52 - 39.52	14.77 - 17.53	89.44 - 103.35

Studies conducted at CICR, Nagpur (Punit Mohan *et. al,* 1997) c showed that the thickness covered by palisade parenchyma per microscopic field ranged between 41.98 to 51.82 µ in immune lines as compared to 37.06µ in 'AKH 4' and 37.48 µ in 'G 27'. However, the area covered by



a: Appearance of grey mildew fungus on leaf of *G.arboreum* cultivar AKH 4. The white symptom spots formed due to raised aerial growth of the fungus resembles curd and hence the disease is known as Dahya/ Dahiya vernacularly.

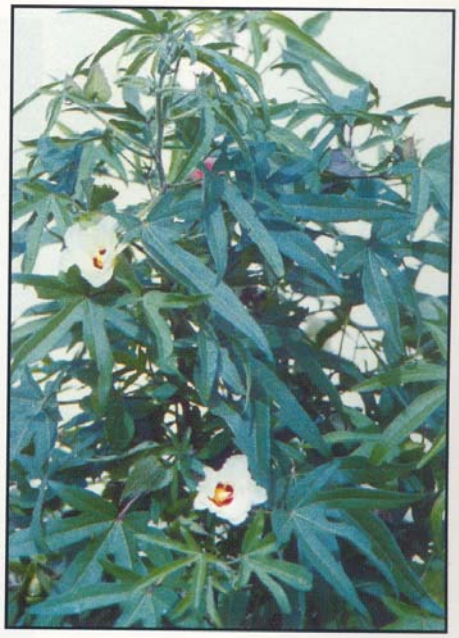
b: Severe intensity of grey mildew disease leads to leaf curling and eventually the defoliation of green leaves.

c: Early appearance of the grey mildew disease on one month old plant of *G.arboreum* cultivar AKH-4.

d: Appearance of grey mildew spots on lower foliage of the *G.herbaceum* cultivar plant Jayadhar

Photo Plate No. 2

(*a*) G-135-49 (INGR 00017)



(b) 30805 (INGR 00018)

(*c*) 30838 (INGR 02020)

Photo Plate No. 3

(a) 30814

(b) 30856

spongy parenchyma showed a single value of 39.52 µ for all the 7 immune lines and susceptible cultivars. Ehrlich and Wolf (1932) conducted histopathological studies in grey mildew affected cotton leaves wherein, they observed the emergence of conidiophores of *Ramularia areola* through the stomata. The conidiophores which bear conidia (spores) in turn arises from a sub stomatal fungus stroma. The fungus hyphae was noticed to ramify from the stroma, coursing both between and within the spongy and palisade parenchyma. Destruction of invaded cells was

accompanied with the advancement of fungus mycelium. Middle lamella was found manifestly dissolved and was evident with separation of attack prone leaf cells. The cells finally were found collapsing completely and disintegrating. Studies at CICR, Nagpur however indicated that the thickness values for palisade parenchyma were always higher in immune lines in comparison to susceptible ones.

CICR studies revealed count for number of epidermal cells per microscopic field between 19.23 to 21.74 in susceptible immune lines whereas it was 14.77 in cultivar AKH 4 and 17.53 in cultivar G 27. All immune lines showed the presence of thick smooth cuticle adhering to the surface of the epidermis and the epidermal cells were closely arranged in a interlocked fashion. Observations for number of stomata per microscopic field indicated a lowest count for all the 7 immune lines ranging from 67.50 to 71.81 as compared to 89.44 in 'AKH 4' and 103.35 in 'G 27'. The stomata were arranged apparently without regular pattern of orientation and were spaced more or less equidistantly from each other. Reporting the pathogenesis of *Ramularia areola* in *G.hirsutum* cotton cultivar HARLL 321-24-73 susceptible to grey mildew, Rathaiah (1977) working in France noticed the host penetration by *R. areola* through the stomata only. In infection process the conidial germ tube was recorded to form an ellipsoidal appressorium (about 13µ long) along the crack between the guard cells, swelling further into a spherical to oval sub-stomatal vesicle (10 µ in diameter) and then giving rise to infection hyphae. The germ tube was also found to enter the open stomata directly occasionally.

Overall results of host anatomical investigations indicated greater variability in the anatomical features of *G.arboreum* immune germplasm lines to grey mildew in comparison to susceptible commercial varieties in *G.arboreum.* The anatomical studies data generated should form a criterion for host resistance parameters in relation to disease immunity.

Land Races in *G.arboreum* and *G.herbaceum*

G.arboreum

Sl. No.	Race	Germplasm Accession (No.)
1	*INDICUM*	793
2	*BENGALENSE*	1010
3	*CERNUUM*	49
4	*SINENSE*	4
5	*BURMANICUM*	13
6	*SOUDANENSE*	1
Available with Germplasm Bank of the CICR, Nagpur		1870 (Total)

G.herbaceum

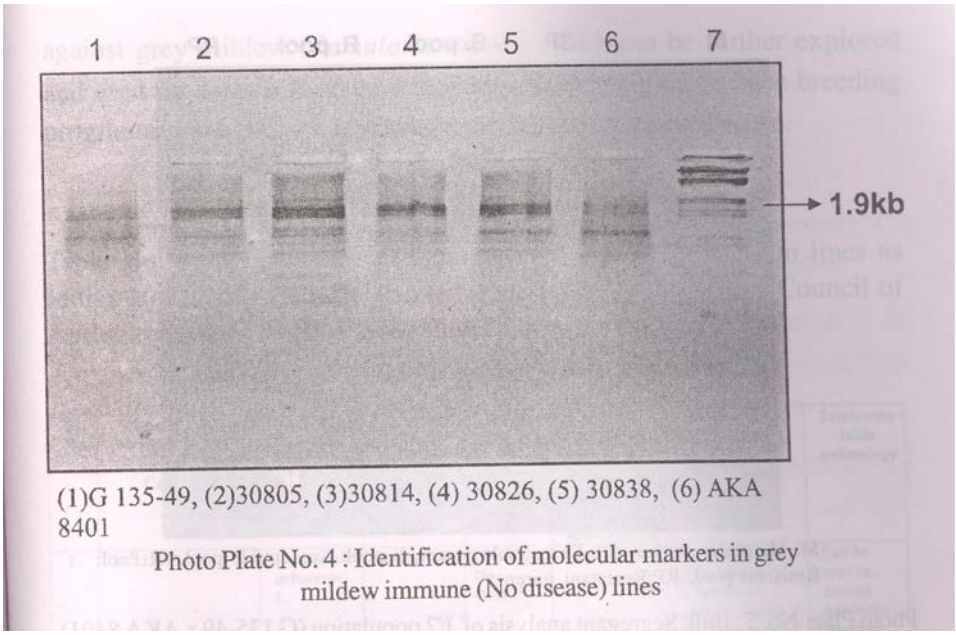
Sl. No.	Race	Germplasm Accession (No.)
1	*WIGHTIANUM*	530
2	*ACERIFOLIUM*	-
3	*PERSICUM*	-
4	*KULJIANUM*	-
5	*AFRICANUM*	-
Available with Germplasm Bank of the CICR, Nagpur		530 (Total)

Molecular Level Studies:

Tagging grey mildew resistant gene in *Gossypium arboreum* by molecular marker

DNA fingerprinting and Molecular Characterization of *Gossypium arboreum* grey mildew immune genotypes 30805, G 135-49, 30814, 30826, 30838 and susceptible *G.arboreum* cultivar AKA 8401, was carried out by molecular marker techniques. (Photo plate No.4)

Genomic DNA was isolated from all the genotypes using Paterson *et.al.* (1994) method. The template DNA of all the genotypes were subjected to PCR amplification using random primers and the products were resolved on 1.5 per cent agarose gel electrophoresis. Genetic similarity and diversity analysis among grey mildew immune lines were performed by UPGMA clustering method for the construction of dendrogram (Bar diagram No.1).

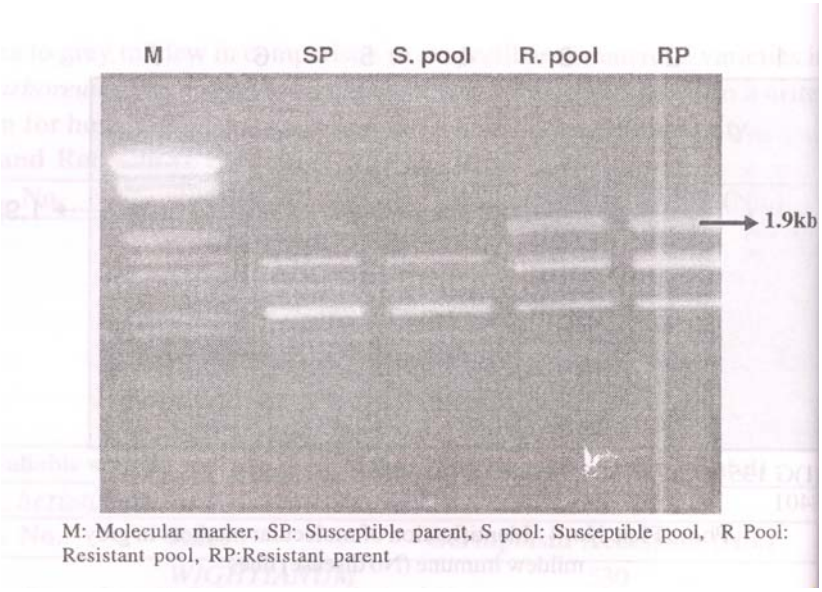


(1)G 135-49, (2)30805, (3)30814, (4) 30826, (5) 30838, (6) AKA 8401

Photo Plate No. 4 : Identification of molecular markers in grey mildew immune (No disease) lines

In resistance breeding programme the grey mildew immune lines were crossed with susceptible *G.arboreum* cultivar AKA 8401 and F2 mapping population was developed. Screening against the disease was done in segregating F2 population wherein phenotypes for resistance were determined by artificial inoculation of conidia of *R.areola* obtained from diseases leaves in water in the green /glasshouse. The chi square test was calculated and observed to fit in 3:1 Mendelian segregating ratio. Bulk segregant analysis (Michelmore *et. al.*, 1991) was carried by pooling ten resistant and ten susceptible DNA pools and subjected to RAPD analysis. The fragment of 1.9 kb was amplified in the resistant pool and resistant parent whereas not in susceptible pool and susceptible parent (Photo plate No.5). Validation of the identified marker was done in the same genetic background. Expected minimum distance of the

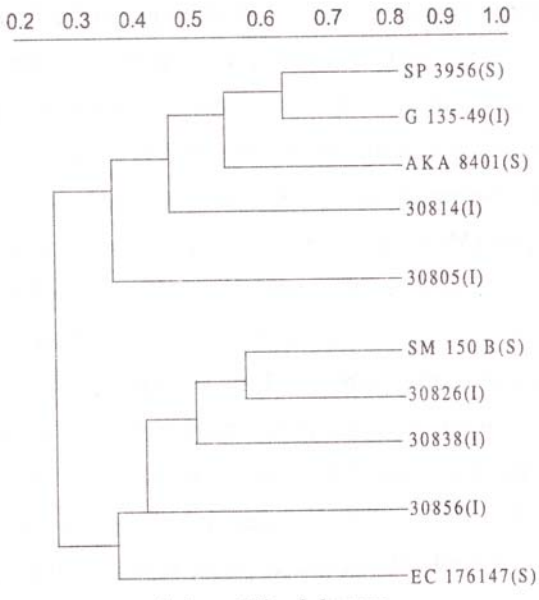
marker from the target gene of interest is 1.9cM (centi Morgan) and the 95 per cent confidence level is 2.19 cM calculated based on the formula by Martin *et.al.*, (1994).



M: Molecular marker, SP: Susceptible parent, S pool: Susceptible pool, R Pool: Resistant pool, RP:Resistant parent

Photo Plate No.5: Bulk Segregant analysis of F2 population (G 135-49 x AKA 8401)

The results of the studies conducted at CICR, Nagpur showed that there is a definite molecular marker reflecting the immunity characterization against grey mildew (*Ramularia areola*) which can be further explored and used for marker assisted selection for developing resistance breeding programme against economically important grey mildew disease.



S: Susceptible, I: Immune

Bar diagram No.1: Dendrogram of the grey mildew immune and susceptible genotypes constructed by UPGMA Method. The scale is based on Nei and Li's coefficient of similarity.

Table No.4: Registration of Immune G.arboreum germplasm lines as Indian National Genetic Resources (INGR) line by the Indian Council of Agricultural Research (ICAR), New Delhi.

Sr.No	Name / Accession Number of CICR Germplasm Catalogue	*Gossypium* species	Race	Registration No.	Plant Germplasm Registration Committee (PGRC) Notification date/ Patient	Unique and Identification characters	Implementable technology
1.	G-135-49	*Gossypium arboreum* L.	*Bengalense*	INGR No. 00017*	10-05-2000	Immune to all grey mildew (*Ramularia areola*) disease isolates existing in nature at present.	Can be used in disease resistance breeding programme
2.	30805	*Gossypium arboreum* L.	*Cernum*	INGR No. 00018*	10-05-2000	Immune to all grey mildew (*Ramularia areola*) disease isolates existing in nature at present.	Can be used in disease resistance breeding programme
3.	30838	*Gossypium arboreum* L.	*Cernum*	INGR No. 02020**	22-05-2002	Immune to all grey mildew (*Ramularia areola*) disease isolates existing in nature at present.	Can be used in disease resistance breeding programme
4.	30814	*Gossypium arboreum* L.	*Cernum*	Proposed for Registration	----------	_____	_____
5.	30826	*Gossypium arboreum* L.	*Cernum*	Proposed for Registration	----------	_____	_____
6.	30856	*Gossypium arboreum* L.	*Cernum*	Proposed for Registration	----------	_____	_____
7.	Bangladesh (EC 174092)	*Gossypium arboreum* L.	*Bengalense*	Proposed for Registration	----------	_____	_____

* Punit Mohan et.al., (2000), Mukewar and Mayee(2001).

** Punit Mohan et.al., (2002)

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