Plant Archives Vol. 25, Supplement 2, 2025 pp. 1312-1320 e-ISSN:2581-6063 (online), ISSN:0972-5210

Plant Archives

Journal homepage: http://www.plantarchives.org
DOI Url : https://doi.org/10.51470/PLANTARCHIVES.2025.v25.supplement-2.162

MORPHOLOGICAL AND BIOCHEMICAL EVALUATION OF EARLY SEGREGATING GENERATIONS FOR LEAF HOPPER RESISTANCE IN UPLAND COTTON

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(Date of Receiving : 24-03-2025; Date of Acceptance : 30-05-2025)

ABSTRACT

The incidence of leafhopper was determined on 354 F_2 plants, 19 F_3 lines, and resistant and susceptible checks. Different morphological and biochemical characteristics of the selected entries were studied in the two generations sequentially over two years. A segregation ratio of 9 (susceptible): 7 (resistant) was shown by the F_2 generation, indicating that two dominant genes with epistatic gene action controlled the leafhopper resistance. Based on Leafhopper resistant index the F_2 population was classified as susceptible. Three lines with an LHRI of 1.5–2.0 were designated as resistant, and two lines were categorised as highly resistant (LHRI: 1- 1.5) amongst the 19 tested F_3 lines. Five lines indicated an intermediate reaction (LHRI: 2.0–2.5). Five lines were highly susceptible, where the injury index was between 3.01 and 4.0, and four lines were designated as susceptible (LHRI: 2.5–3.0). In comparison to susceptible plants, the resistant plants had low mean values for leafhopper number, total sugars, and reducing sugars, and high mean values for trichome density, trichome length, phenols, tannins, gossypol and lignin in both generations. Total phenols, tannins, trichome density, and trichome length all had negative and significant correlation with the injury grade; gossypol and lignin content in the F_2 generation had negative but non-significant correlations. The association between leafhopper injury grade with leafhopper count, total sugars, and reducing sugars was significant and positive. These host plant resistance traits could be used against leafhoppers in subsequent breeding programs to come up with insect-resistant cotton genotypes.
Keywords: cotton, leafhopper, leafhopper injury grade, segregating generations, gene action.

Introduction

Cotton (*Gossypium* spp.), one of the world's most important fiber crops, commonly referred to as the "King of fiber" or "White Gold", is one of the most valuable commercial crops and has a central role in the economic, social and political position of the world. India was known as the cradle of the cotton industry for more than a thousand years (1500 BC to 1700 AD). India is the second largest producer of cotton in the world and owns about 25 per cent of the total area of cotton in the world and 22 per cent of cotton production worldwide. India has the largest area of cotton among countries in the world, yet cotton yield is poor with the average yield being 461 kg/ha against the world average of 854 kg/ha (ICAR AICRP on cotton annual report 2024-25). Therefore, there is a need to genetically enhance the productivity of cotton, as it holds significant economic and social importance for the Indian farming community.

Cotton's ecological environment supports nearly 162 insect pest species and the yield loss because of the insect pests is estimated at Rs. 2,87,000 million annually (Avinash *et al.*, 2022). Cotton suffers significant losses because it is prone to insect pests such as bollworms and sucking pests. The newly introduced Bt cotton hybrids are resistant to bollworms but the Bt toxins are not effective against sucking pests, particularly leafhoppers (Kranti and Stone, 2020). The yield loss in seed cotton attributable to leafhopper infestation alone has been estimated at approximately 390 kg/ha before the development of Bt cotton (Pandi, 1997). Presently, losses are even greater since the sucking pest complex has become the scourge of Bt cotton. The infestation of the sucking pest, *Amrasca biguttula biguttula*, also referred to as *Amrasca devastans* (Dist.), has turned into a serious problem in India (Singh and Agarwal, 1988; Manivannan et al., 2017) as well as in other countries of South East Asia. It dominates throughout the vegetative phase through the reproductive phase of crop growth. Both adults and nymphs cause injury by sucking the sap of leaves resulting in yellowing, reddening and leaf drying characteristic of phytotoxaemia known as "hopperburn" (Painter, 1951; Uthamasamy, 1985) causing significant yield loss. Hence, development of leafhopper-resistant high-yielding genotypes become crucial.

Insect resistance in cotton is linked with various morphological, and biochemical characteristics. The research on resistance mechanism disclosed that leafhoppers differentiate between cotton genotypes for settling and feeding. The highly susceptible types are settled and fed up on preferentially, while varieties that are less preferred for settling are likewise less preferred for oviposition (Murugesan and Kavitha, 2010). Antixenosis (where insect may avoid less damaged plant) and antibiosis (less hospitable as a host) are postulated as resistance mechanisms. The non-glandular trichomes hinder the movement of insects and other tiny arthropods across the surface of the plant, hindering insects' ability to reach the leaf epidermis below to feed (Southwood, 1986). Also, the cotton plant possesses a complex array of phytochemicals, which serve as repellents, phagodeterrents, and oviposition deterrents, all demonstrating resistance. In the current research program, an attempt has been made to investigate the genetic inheritance pattern of resistance to leafhoppers and the morphological and biochemical basis of resistance to leafhopper injury in American cotton.

Material and Methods

The experimental material consisted of early segregating generations (F_2 and F_3) developed from the selfing of F_1 of the cross, Raider 276 × 8-1-2. Raider 276, originating from the United States of America, possesses very few trichomes on the abaxial surface of the leaf, making it highly susceptible to leafhopper attacks with a leafhopper injury grade of IV. However, it has big bolls with a hard boll rind and exhibits good fiber properties. In contrast, 8-1-2, bred at Raichur in Karnataka, India has moderate trichome density on the abaxial surface of the leaf, rendering it highly resistant to leafhopper attacks with a leafhopper injury grade of I. This genotype produces medium-sized bolls. The segregating generations (F_2 and F_3) were evaluated during the kharif- 2023-24 and 2024-25 at Agricultural Research Station, Dharwad, Karnataka, India. A total of 354 F_2 plants along with leafhopper resistant (DLSA-17) and susceptible check (CPD - 1401), were assessed for field incidence of leafhopper. Nineteen F_3 lines were selected from F_2 plants and were evaluated for leafhopper incidence during kharif, 2024-25. The package of practices recommended for cotton cultivation under assured rainfed conditions minus the plant protection protocol, was followed.

Morphological Observations

Leafhopper count was taken on each F_2 and F_3 plant's three leaves, one each in the top, middle, and bottom portions of every plant). The observations were recorded two times, the first in mid-August and the second time in October during which leafhopper incidence was at peak or its ETL *i.e*., 2 nymphs/ leaf. The density of nymphs was counted visually by observing the abaxial surface of the leaves. The average value from the three leaves per plant was taken for analysis. Leafhopper injury grade was recorded on each plant of both generations following the criteria provided by the Indian Central Cotton Committee (ICCC) (Table 1). Based on the observations of hopper burn injury grade symptoms and leafhopper count on each plant in both generations, the leafhopper resistance index (LHRI) was calculated according to the formula given by Nageswara Rao (1973).

$$\mathrm{LHRI} = \frac{G1 \times P1 + G2 \times P2 + G3 \times P3 + G4 \times P4}{P1 + P2 + P3 + P4}$$

where, G – Leaf hopper Injury Grade of ICCC (Sikka *et al.*, 1966 and Nageswara Rao, 1973), P - The plant population under the grade for each category. After indexing, the entries were categorized as highly resistant (1.0 - 1.5), resistant (1.51 - 2.0), intermediate (2.01 - 2.5), susceptible (2.51 - 3.0) and highly susceptible (3.01 - 4.0), following Pandi (1997).

Based on the segregation pattern of F_2 populations for leafhopper infestation, the genetic basis determining the resistant phenotype in the host plant was confirmed by chi-square test (goodness of fit)

$$X^2 = \sum \frac{(O - E)^2}{E}$$

Where, O = Observed frequency

E = Expected frequency

The chi-square values were compared to table values at n-1degrees of freedom (n being the number of classes) or the probability value was used to determine if the null hypothesis should be rejected or accepted.

Measurements of trichome density and trichome length on the abaxial surface of the leaf were taken. Two leaves from the 5th node from the top of the plant were taken at 60 days after sowing, cut into 1cm^2 discs throughout the leaf, and placed in a solution of acetic acid and ethanol (2:1) for 24- 48 hours. Lactic acid as a clearing agent is utilized for enhancing the visibility of trichomes. Samples were then examined using a high-resolution Olympus BX41 microscope for the number and length of trichomes. Five plants for each grade and each line of F_2 and F_3 respectively, were taken for the observation of trichomes. Calculations were made based on the average of five plants.

Biochemical Studies

To uncover the biochemical basis of leafhopper resistance, biochemical constituents like phenols, tannins, total sugars, reducing sugars, and gossypol were estimated in the unprotected (no pesticides spray) crop at 60 days after sowing as per Sadasivam and Manickam (1991). Lignin was measured as per Fukushima and Kerley (2011). Leaf biochemical analysis was conducted on five plants from each grade and line in the F_2 and F_3 generations, respectively. The calculation was done with the average of five plants.

Results

The frequency distribution for leafhopper injury grade in the F_2 population is depicted in Figure 1. Among the 354 F_2 plants studied, 140 plants exhibited the resistant phenotype (grade I or II) comparable to the resistant check (DLSA-17), while 214 plants showed susceptible reaction (grade III or IV) comparable to the susceptible check (CPD-1401) with grade IV (Fig. 1). Based on the LHRI, the F□ population was classified as susceptible, with a value of 2.58. Based on the segregation pattern of the F_2 population, various epistatic interactions and their standard ratios were analyzed. The studied population data was certainly fitted into the modified dihybrid ratio 9:7 (9 susceptible, and 7 resistant types), confirmed by the chi-square test for goodness of fit (Table 2). The calculated chi-square value was less than the critical value (3.84) at a 5 % level of significance with one degree of freedom which revealed that there was no significant difference between the observed and expected ratio, and the deviations observed in the studied populations were due to chance factor. During the next season, 19 F_2 plants belonging to different grades were selected and advanced to the next generation as progeny rows and were evaluated for leafhopper infestation along with resistant and susceptible checks.

Two of the 19 F_3 lines tested were highly resistant according to the LHRI (1 to 1.5). Three lines were resistant with an average of 1–11 leafhoppers per three leaves and a LHRI of 1.5 to 2.0. Five lines were intermediate in reaction with an LHRI of 2.0 to 2.5. Four lines were susceptible, with an injury index of 2.5–3.0, and the remaining five lines were very susceptible, with an injury index of 3.01–4.0 (Table 3).

The mean values of morphological and biochemical traits in the F_2 population are given in table 5. Trichome analysis revealed that tolerant plants (Grade I & II) had a higher trichome density ranging from 314.63 to 341.25 in F_2 generation and 296.98 to 301.32 in F_3 generation, as found in resistant check DLSA-17. Trichome density was negatively and significantly associated with leaf hopper injury grade in both generations (Table 4). In the susceptible plants (Grade III & IV), trichome density ranged from 109.33 to 327.51 in the F_2 generation and 198.78 to 287.15 in the F_3 generation. Trichome length also differed considerably between the resistant and the susceptible plants ranging from 1269.58 to 2047.42 µm in resistant plants and from 696.87 to 1240.04 µm in susceptible plants. Trichome length was also significantly negatively correlated with leafhopper injury grades in both the generations (Table 4).

Biochemical Analysis

F_2 population: Biochemical analysis revealed clear differences among susceptible and resistant genotypes. The highest concentration of phenol was found in leaf hopper injury grades I (11.86 mg/g) and II (11.91 mg/g), while phenol content at lower levels occurred in plant injury grades III (5.45 mg/g) and IV (4.66 mg/g). This was also true of tannins, which showed the most abundance in grades I and II (5.43 and 4.4 mg/g, respectively) and the least in grades III and IV (1.66 and 2.95 mg/g, respectively). Both phenols and tannins exhibited a strong significant negative correlation with leafhopper injury grades. On the other hand, total sugars and reducing sugars, both presented strong positive correlations with leaf hopper infestation. The lowest levels of sugars were detected in grades I and II (total sugars: 4.33 mg/g; reducing sugars: 0.86 mg/g), while the highest found in grades III and IV (total sugars: 19.42 mg/g; reducing sugars: 2.545 mg/g). The average contents of gossypol and lignin were 0.315 and

3.24 mg/g, respectively, in grades I and II, and 0.28 and 2.79 mg/g in grades III and IV. The relationship of gossypol and lignin with leafhopper injury grade was negative and nonsignificant (Table 4).

F_3 population: The same trend was also found in F_3 lines for biochemical contents. The mean phenol (10.01 mg/g), tannin (5.33 mg/g) and gossypol (0.41) contents were the highest in highly resistant and the lowest in highly susceptible lines. Concentrations of total sugars and reducing sugars were high in susceptible lines. Correlation analysis showed a significant and strong negative correlation between phenol and tannin content with leafhopper injury grade (Table 4). Conversely, reducing sugars and total sugars had a strong positive correlation with leafhopper damage, indicating possible involvement in susceptibility. The correlation of gossypol and lignin with injury grades of leafhoppers was negative and significant.

Discussion

Several plant characteristics are accountable for resistance to insect pests. Trichomes, for example, are important in determining insect oviposition and feeding behaviour (Levin, 1973; Madhu and Mohan, 2021). Non-glandular trichomes are largely a structural defense against small herbivores, inhibiting insect movement and making it hard for them to reach the leaf epidermis to feed (Nandi *et al.*, 2022). Made primarily of cellulose and other low-nutritional-content materials, trichomes can suppress insect weight gain and enhance mortality. Leaf trichome density and length greatly impact the host-plant choice, as well as the growth, survival, and reproduction of herbivorous insects. Besides structural defence, biochemical components are key in pest resistance. Phenols, for instance, cause precipitation of proteins like the salivary enzymes of insects, thus inhibiting their capacity to degrade plant tissues (Pratyusha, 2022). On the other hand, sugars are a major source of energy for all living things and can induce feeding behaviour in leafhoppers. Gossypol, which is a secondary metabolite, acts as a leafhopper deterrent (Banoth *et al.*, 2023). It has been confirmed that the development of insects, their survival, and reproduction are greatly affected by the overall content of amino acids in their food (Srivastava & Auclair, 1974).

The parents chosen for this study showed wide variation in leafhopper injury response. The exotic parent, Raider 276, was very susceptible (injury grade: IV), while 8-1-2 was tolerant (injury grade: I). The F_2 progeny from these parents were segregated into two clear classes, following a 9:7 susceptible-to-resistant ratio, consistent with complementary gene interaction (duplicate recessive epistasis). This indicates that two genes are involved in resistance and at least one dominant allele from each of them must be present for the expression of the resistance trait. If either gene is homozygous recessive, it overcomes the other dominant allele to cause susceptibility. These results confirm the investigation of Pushpa & Raveendran (2005) in upland cotton, where the same digenic segregation ratios were reported in different F_2 crosses. Similar findings have similarly been documented by Mahal (1978), Radhika *et al.* (2004), Murugesan & Kavitha (2010), Zhang *et al.* (2013), Venkatesha (2014), and Yaksha *et al.* (2022).

All the resistant lines were on par with a resistant check (DLSA-17), which had a mean of 0.48- 0.67 leafhoppers/3 leaves with an injury index of one. The susceptible checks (CPD-1401) had an average of 8.25- 9.63 leafhoppers/3 leaves with the leafhopper injury index of four (Table 3). Similar results were demonstrated by other studies (Dhillon and Sharma, 2013; Manivannan *et al.*, 2017; Patel and Radadia, 2018; Sasikumar and Rathika, 2020; Sivaram Krishna and Rama Reddy, 2020; Avinash *et al.*, 2022; Gangavati and Maralappanavar, 2022; Senguttuvan *et al.*, 2022).

In both the F_2 and F_3 generations, the leafhopper population was positively and significantly correlated with injury grade, a trend consistent with earlier studies (Tikade & Sane, 1962; Ali *et al.*, 1995; Uthamasswamy *et al.*, 1985; Mohankumar, 1996; Syed *et al.*, 1996; Khan *et al.*, 2001; Murugesan & Kavitha, 2010; Madhu *et al.*, 2024). Tolerant plants in these populations exhibited higher mean trichome density and trichome length, values comparable to the resistant checks. The strong negative correlation between leafhopper injury grade and both trichome density and length, suggests that increased trichome presence plays a crucial role in resistance by hindering insect movement and feeding. Similar findings have been reported in previous studies, confirming that trichome density negatively correlates with leafhopper infestation (Rustamani *et al.*, 2014; Sandhi *et al.*, 2017; Manivannan *et al.*, 2021). Observation from line 225, suggests that a greater trichome length can contribute to resistance even if the density is comparatively low, indicating a potential compensatory mechanism (May, 1951).

In addition to trichomes, phenol, and tannin contents were significantly higher in resistant lines compared to susceptible ones. These compounds showed a negative correlation with leafhopper injury grade, indicating their role in resistance, possibly through anti-feedant or toxicity mechanisms. Phenols are known to act as repellents against herbivores by precipitating pest salivary enzymes, thereby limiting tissue penetration and subsequent feeding (Wink *et al.*, 1972; Nelson *et al.*, 1983; Rhodes, 1979). Similar findings have been reported by Chan *et al.* (1978), Thimmaiah (1992), and Rohini *et al.* (2011), sandhi *et al.* (2017), Rizwan *et al.* (2021), Banoth *et al.* (2023), Vinutha *et al.* (2023) and Mawblei *et al.* (2024). Conversely, sugar content exhibited a significant positive correlation with the leaf hopper population, supporting the hypothesis that increased sugar availability enhances plant susceptibility (Rana & Manzoor, 1990; Thimmaiah, 1992; Nizamani *et al.*, 2002; Iqbal *et al.*, 2011; Sandi *et al.*, 2017, Rizwan *et al.*, 2021; Vinutha *et al.*, 2023). The present study confirms that high sugar levels contribute to leafhopper attraction and infestation. Although the gossypol content in cotton leaves was lower than in seeds, it exhibited a negative association with leafhopper infestation (Irfan *et al.*, 2010, Madhu *et al.*, 2024). However, this correlation was not statistically significant in the F_2 population. Despite its role as a leafhopper repellent (Rana & Manzoor, 1990), the present study suggests that gossypol's contribution to resistance may not be as pronounced as other biochemical factors. It was also noted that in F_3 lines, 124 and 165, even though trichome density was comparatively less, the lines showed resistant reaction. This implies that biochemical compounds such as phenols and tannins and lower amounts of sugars might be conferring resistance. Similarly, the association between lignin and leafhopper infestation was negative and significant in F_3. These results contrast with the findings of Iqbal *et al.* (2011) and Sandi *et al.* (2017), who reported a strong positive association between leafhopper infestation and lignin content.

Conclusion

Field screening of early segregation populations for morphological and biochemical analyses was carried out at 60 DAS during the critical period of pest activity. The genetic study revealed a 9:7 digenic segregation ratio, which indicates complementary gene interaction for leafhopper resistance. The present research exhibits that both the morphological and the biochemical traits are involved in the resistance of cotton leafhoppers. Increased trichome length and density, and higher levels of phenol, tannin, gossypol and lignin are associated strongly with resistance. Conversely, higher sugar induces susceptibility by an increasing leafhopper feeding response. This study is among the few comprehensive efforts to integrate both morphological and biochemical traits for dissecting leafhopper resistance in an F_2 population of cotton and these results offer useful information for breeding programs to produce leafhopper-resistant varieties of cotton.

Table 1: Leafhopper injury grading according to ICCC

Grades	Symptoms
1	Leaves free from crinkling or with no yellowing, bronzing and drying
2	Few leaves on lower portions of the plant curling, crinkling and slight yellowing
3	Crinkling and curling all over, yellowing, bronzing and browning in the middle and lower portion, plant growth hampered
4	Extreme curling, yellowing, bronzing and browning, drying of leaves and defoliation, stunted growth

Table 2 : Segregation Pattern of F_2 populations for leafhopper

Cross		Reaction to leafhopper		Total	χ2 value (test statistic)	χ2 value (critical value)	expected ratio	p
		Susceptible (grade III & IV)	Resistant (grade I &II)					
Raider 276 × 8-1-2	O	214	140	354	2.53	3.841	9:7	0.05
	E	199.12	154.87					

O-observed values, E- expected values, Level of significance(α) =0.05, Degrees of freedom = 1

Table 3 : Field and biochemical evaluation of selected F_3 progeny rows (along with parents and checks) for Leafhopper

F_3 lines	Leafhopper population range/3 leaves	LHRI	Phenotype	Phenols* (mg/g)	Tannins* (mg/g)	Total sugars* (mg/g)	Reducing sugars* (mg/g)	Gossypol* (mg/g)	Lignin* (mg/g)	Trichome density*	Trichome Length* (um)
3	2- 9	2.34	I	5.56	3.14	7.63	2.24	0.31	2.64	254.53	1101.60
31	0-8	1.44	HR	10.01	5.33	6.35	0.57	0.33	3.43	296.98	1525.38
39	5-10	3.76	HS	3.12	0.81	8.98	4.99	0.09	2.61	219.84	1030.34
65	1- 12	2.85	S	3.66	1.06	9.13	4.82	0.12	2.18	200.26	898.69
79	2- 16	2.76	S	2.93	1.99	7.39	5.27	0.21	3.51	198.87	974.30
85	5- 9	3.1	HS	2.09	1.62	7.92	5.06	0.26	1.09	228.91	1179.94
89	3- 11	2.33	I	4.86	2.71	5.68	3.19	0.22	3.65	251.36	1365.98
112	2- 6	2.41	I	5.01	3.55	6.91	2.92	0.16	3.13	245.87	1295.26
113	5-12	3.82	HS	3.88	2.82	9.02	4.02	0.18	3.26	209.28	820.90
115	6- 10	4	HS	4.73	3.19	7.18	5.18	0.20	3.25	227.85	1047.20
118	6- 12	3.91	HS	3.65	2.93	6.88	6.28	0.18	2.48	201.56	867.14
124	1 – 5	1.69	R	8.01	3.65	2.65	3.92	0.12	2.39	239.02	993.89
129	4-11	2.82	S	3.85	2.81	6.31	3.09	0.26	3.14	287.15	915.55
130	4-14	2.45	I	5.02	3.92	6.99	3.18	0.29	4.22	234.75	1166.34
164	2-13	3	S	5.21	3.88	8.03	5.66	0.19	2.38	235.63	1062.43
165	3-7	1.82	R	9.36	3.16	4.51	4.19	0.25	1.18	237.27	914.46
209	1- 11	1.76	R	5.19	3.71	6.92	2.01	0.41	4.01	301.32	1398.62
225	4- 12	1.31	HR	4.92	2.23	5.09	4.94	0.33	3.55	298.14	1464.45
233	1-6	2.37	I	4.98	2.71	3.92	4.11	0.12	3.18	270.35	1033.06
Raider 276	6-14	4	HS	1.94	1.93	9.47	6.82	0.26	2.73	224.74	920.45
8-1-2	2-6	1	HR	7.72	4.67	4.25	1.02	0.29	3.51	433.82	1432.35
DLSA-17	0-4	1	HR	6.68	5.16	1.93	0.88	0.41	3.38	429.87	1334.43
CPD-1652	3-15	4	HS	3.02	1.18	8.79	3.99	0.18	2.05	215.67	1143.49

HR-highly resistant, R-resistant, I-intermediate, S-susceptible, HS-highly susceptible

* average of five plants in each line

Table 4: Correlation of Leafhoppers injury grade with biochemical parameters in F_2 and F_3 population

Segregating generation		Leafhopper count	Phenols	Tannins	Total sugars	Reducing sugars	Gossypol	Lignin	Trichome density	Trichome length
F_2	Leafhopper injury grade	0.71*	-0.92**	-0.80*	0.87*	0.86*	-0.35	-0.25	-0.99**	-0.83**
F_3		0.64**	-0.81**	-0.68**	0.22	0.83**	-0.48*	-0.59**	-0.65**	-0.68**

*p<0.05, **p<0.01

Table 5: Mean values of morphological and biochemical traits in the F_2 population

Leafhopper injury grade	Leafhopper count	Phenols	Tannins	Total sugars	Reducing sugars	Gossypol	Lignin	Trichome density	Trichome length
I	3.26	11.86	5.43	5.78	1.04	0.26	3.26	341.25	1263.91
II	8.08	11.91	4.40	2.88	0.68	0.37	3.21	314.63	1098.74
III	12.15	5.45	1.66	18.46	2.43	0.19	2.37	288.72	1182.27
IV	17.14	4.66	2.95	20.39	2.66	0.25	2.19	239.18	921.36

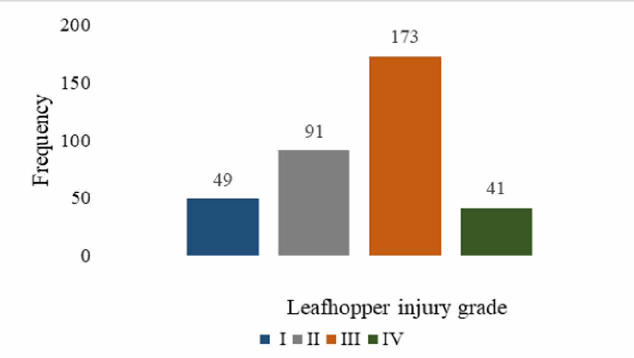

Fig. 1: Frequency distribution for leafhoppers injury grade in F_2 population

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