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Standardization of screening techniques for resistance to *Lipaphis erysimi* (Kalt.) in rapeseed-mustard under field conditions

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The population and damage by aphid, *Lipaphis erysimi* (Kalt.) in *Brassica* spp. is highly variable across seasons and regions, wherein screening of rapeseed-mustard genotypes under natural infestation conditions has not been rewarding for aphid resistance. Since no reliable screening technique is in place, we developed and evaluated various screening techniques to differentiate diverse mustard genotypes for resistance to *L. erysimi* under field conditions. Artificial infestation at bud formation stage with 20 mixed stage aphids pinned with bell pins on the top third branch near inflorescence was found most appropriate and effective for establishment of aphids at inoculation site. Evaluation of mustard genotypes under multi-choice natural infestation revealed maximum variability in *L. erysimi* resistance indices, but plot cage artificial screening technique was found appropriate over natural infestation for multi-choice assays. Genotypes Heera and PDZM 31 showed susceptible to highly susceptible reaction against *L. erysimi* under all the artificial infestation screening techniques. However, PM 30, PM 21, Pusa Bold and Pusa Vijay displayed variable resistance reactions under different screening techniques. Although no-choice twig cage and plant cage techniques showed significant differences in test mustard genotypes for various aphid resistance indices, the twig cage technique revealed maximum variability and could differentiate them at slightest variation in levels of tolerance/susceptibility to *L. erysimi*. The rate of *L. erysimi* multiplication on test mustard genotypes was highly variable under plant cage as compared to twig cage. The twig cage technique also successfully differentiated the double low erucic acid and total glucosinolate, single low erucic acid, and conventional varieties with high erucic acid and total glucosinolate groups of mustard genotypes for *L. erysimi* resistance. The multiplication rate and ease in scouting of aphids, easy handling and cost of the cage, and natural plant growth conditions are some of the most favourable factors, suggesting twig cage technique more précised, realistic, economical, and efficient for artificial screening of rapeseed-mustard for resistance against the aphid *L. erysimi* infestation.

Keywords: Aphids resistance screening, *Brassica*, Mustard aphid, Twig cage technique

Rapeseed-mustard is one of the most important oilseed crops in India, which occupies third place after soybean and palm in the world[1]. In India, it occupies second position in edible oilseed production after groundnut contributing to about 27.8% of the Indian oilseed economy[2]. It is grown on an area of 5.8 million ha with production of 6.28 million tons in India[3]. The average productivity of rapeseed-mustard in India (1176 kg/ha) is about two-third of world's (1695 kg/ha) average yield[2]. Mainly four oleferous rapeseed-mustard species viz., *Brassica juncea*, *B. rapa*, *B. napus* and *B. carinata* are grown under different agro-climatic conditions in India, of which *B. juncea* is the frontrunner occupying >80% of rapeseed-mustard area[4]. Production and productivity of rapeseed-mustard is highly variable across crop growing states of India due to variable agroclimatic conditions, cropping systems, production technologies, biotic and abiotic stresses. Among the biotic stresses, mustard aphid, *Lipaphis erysimi* (Kalt.) is considered as most important pest of cruciferous crops worldwide[5-8], and is a major constraint for the production of rapeseed-mustard across agro-ecological region of India. The damage by *L. erysimi* vary from 10-90% depending upon the climatic conditions, intensity of population build up and crop growth stage[9]. Mustard aphid, *L. erysimi* is a specialist *Brassica* feeder, and the oviparity and viviparity modes of reproduction makes it important and difficult to control them. Oviparity in *L. erysimi* is for migration and host finding, while viviparity mode of reproduction for establishment and multiplication. Shorter generation time, parthenogenetic viviparity and higher multiplication, and population growth rates make it economically most significant and important pest of rapeseed-mustard[10].

Mustard aphid is not only a major threat to Indian rapeseed-mustard, it has also been reported one of the

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most destructive pest in other rapeseed-mustard growing countries Bangladesh and Pakistan[11-14]. Both the adults and nymphs cause damage to mustard at vegetative, flowering and pod formation stages by sucking sap from the plant. Continuous aphid feeding inhibits plant growth resulting in poor pod formation, less seed set, low oil content, and reduced seed yield[15,16]. *L. erysimi* has also been reported to transmit about 13 different viruses, including important viruses of the Brassicaceae, such as *Beet mosaic virus*, *Cabbage black ring spot virus*, *Cauliflower mosaic virus* and *Radish mosaic virus*[17,18]. In case of severe infestation, leaves become curled, plant fails to develop pods, and young pods if developed do not mature and produce unhealthy seeds. Although several aphid management tools like adjustment of sowing dates, yellow sticky traps, biological control, etc. are being talked about, but it is currently being managed by insecticide applications. The insecticide sprays not only increase cost of cultivation, but also disrupt the aphid-natural enemy balance, pollination services, and leave harmful residues in the food and the environment[19]. To minimize insecticide use an alternate, effective, economic and environmentally safe method of aphid management is highly desirable. In this context, development and cultivation of *L. erysimi* resistant rapeseed-mustard varieties gains attention as it imparts inherent insect control.

Rarely a researcher is able to evaluate and interpret insect damage accurately under natural infestation as either there are insufficient insect numbers to cause adequate damage or insects occur at an inappropriate phenological stage of the crop growth[20]. Therefore, standardization of an efficient and reliable screening technique is highly desirable for identification of sources of resistance and evaluation of breeding materials for resistance to insect pests. Earlier efforts of rapeseed-mustard evaluation against aphids indicate that the sources of resistance in the primary gene pool are rare bearing low to moderate levels of resistance to *L. erysimi*[21-25]. These efforts have relied on natural infestation conditions, wherein several factors interplay to counterfeit in identification of resistant/tolerant rapeseed-mustard genotypes. Moreover, the variation in population and damage by *L. erysimi* in rapeseed-mustard across seasons and regions, and influence of environmental factors on it's biology and behavior, has made it difficult to identify reliable sources of resistance under natural infestation conditions. No reliable screening technique has been developed yet which can differentiate aphid resistant genotypes from susceptible ones. Thus, it was highly desirable to develop/standardize a dependable screening technique to evaluate rapeseed-mustard for resistance to *L. erysimi* under field conditions. Therefore, we developed and evaluated different artificial screening techniques for their efficiency in differentiating diverse mustard genotypes for resistance to *L. erysimi* under field conditions.

Materials and Methods

The experiments were conducted at ICAR-Indian Agricultural Research Institute, New Delhi (Latitude 28°38'23" N and Longitude 77° 09'27"E, height above mean sea level 228.61 meters) during 2013-15 *Rabi* (winter) seasons under field conditions. We standardized artificial aphid infestation method and evaluated diverse mustard genotypes using different cage screening techniques for their appropriateness to use in identification of sources of resistance to *Lipaphis erysimi*, and further deployment in aphid resistance breeding program.

L. erysimi culture and standardization of artificial infestation technique

Aphids can be reared in large numbers on natural food in the laboratory or can be directly obtained from the field to inoculate the test plant material to ascertain uniform insect infestation. Standardization of a technique to infest the material at the susceptible stage with uniform insect density is essential for successful evaluation of the test plant material under artificial infestation conditions. While devising a technique to inoculate the plant material, it is important to take into consideration of (i) the stage of the insect and inoculation procedure; (ii) the number of insects and time of inoculation; and (iii). susceptible stage of the crop and the site of inoculation. It is well known that rapeseed-mustard is more prone to *L. erysimi* damage from bud initiation to pod formation stages, resulting in extensive yield loss. Our efforts to standardize susceptible stage and appropriate plant site revealed that the bud formation stage is most appropriate which enable aphids to hide and establish on the mustard plant. Since, both nymphs and adults are damaging stages and exit together on the plant, we found that mixed stages of *L. erysimi* are more appropriate to inoculate on the mustard plants. *Lipaphis erysimi* is easily available in enough numbers on rapeseed-mustard crop in the field during this season, thus we used field collected aphids for artificial infestation on test mustard genotypes. We

compared two infestation methods, (i) inoculation with 10 individual *L. erysimi* adults transferred with camel hair brush; and (ii) the *L. erysimi* infested mustard twigs cut into small pieces accommodating around 20 mixed stage aphids (nymphs and adults) were pinned to the inoculation site with the help of bell pins (Nickel plated, solid head, needle point pins) on the third branch from top of the rapeseed-mustard plant. The ease of inoculation and establishment of aphids on the site of inoculation were taken as criteria for selection of appropriate inoculation technique. Transfer of individual aphids with camel hair brush was difficult to handle, time consuming, and experienced with poor aphid establishment. Conversely, the inoculation through aphid infested twigs was found easy to handle and the establishment/transfer of aphids on the site of inoculation was found effective and satisfactory. Thus, we used aphid infested mustard twig method of artificial inoculation for further studies.

Plant materials

We used six diverse mustard genotypes *viz.*, Heera and PDZM 31 (double zero low in erucic acid and total glucosinolate), PM 21 and PM 30 (low in erucic acid only), and Pusa Bold and Pusa Vijay (conventional varieties high in erucic acid and total glucosinolate) procured from Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi. We planted four sets of these test genotypes one each for the test screening techniques in randomized complete block design in three replications. The seeds of test mustard genotypes were sown in four row plots of 5 meter row length having 15 cm plant-plant and 30 cm row-row spacing during 2013-15 *Rabi* seasons under field conditions. The crop was raised following all the recommended cultivation practices, except insecticide application.

Cage screening techniques

The population and damage by aphid, *L. erysimi* in rapeseed-mustard varies across seasons and regions, and is difficult to identify genotypes with resistance to this pest under natural infestation conditions. Caging the test plants with insects is another dependable method of screening for insect resistance under field conditions. (Fig. 1A). Under caging, considerable control is exercised on maintaining the uniform insect

Fig. 1 — Glimpses of different screening techniques: (A) Overview of no-choice caging techniques (Inset: Plant and Twig cages); (B) Twig cage technique; (C) Plant cage technique; (D) Plot cage technique; and (E) Open field natural infestation technique.

pressure on the test genotypes, and plants are inoculated at the same phenological stage. This method of screening also protects the test insect from natural enemies and prevents it's migration away from the test plants. Therefore, we designed and evaluated two cage techniques for no-choice (twig cage and whole plant cage), and two techniques for multi-choice (artificial inoculation in plot cage and open natural infestation) conditions for their effectiveness to screen rapeseed-mustard genotypes for resistance to *L. erysimi* under field conditions (Fig. 1A). Further, the evaluation criteria and resistance indexing proposed by earlier workers[19,20] was found unsuitable in terms of capturing plant stage-specific *L. erysimi* population build up and translation in to plant damage and yield loss. Thus, we modified and used this newly developed evaluation system encompassing various aphid population and damage indices to categorize rapeseed-mustard genotypes for resistance to *L. erysimi* (Table 1).

No-choice cage screening techniques

Two types of no-choice cages *viz.*, cylindrical twig cage (15 cm diameter × 60 cm height mounted on 3 rings one each at top, middle and bottom of the structure; Fig. 1B) and whole plant cage (45 cm diameter × 90 cm height mounted on 3 rings one each at top, middle and bottom of the structure; Fig. 1C) were designed using three ring light muslin cloth bag stitched at one end and open at another end. Quality of muslin cloth were such that air and light can easily penetrate the cage, and normal photosynthetic activity takes place in the plant and conducive for the growth and establishment of the aphids. Ten randomly selected plants of each test mustard genotype from middle two rows in each replication were tagged and inoculated using aforesaid inoculation method, i.e., *L. erysimi* infested mustard twig method of artificial infestation at bud initiation stage for both no-choice cage screening techniques. Before inoculation it was ensured that there were no aphids and other non-target insects including natural enemies on the test plants. The third branch from the top of each test plant was inoculated with aphids for both twig and whole plant cage techniques. After inoculation with *L. erysimi*, in case of twig cage technique only the inoculated twig was covered with twig cage; while in whole plant cage technique, full test plant was covered with plant cage. The lower ends of the cages were tied together to close entry or exit of aphids and other non-target insects. The plant and twig cages were hooked on the bamboo sticks (to provide support) as per the desired height and fixed in the soil near test plants.

Multichoice screening techniques

For multi-choice evaluation of mustard genotypes for resistance against *L. erysimi*, we used two screening techniques *viz.*, plot cage and open natural infestation conditions. For multi-choice plot cage technique, we designed a plot cage comprising of iron pipes (6 m length × 5 m width × 2.7 m height; Fig. 1D) clamped together to make the structure and covered with fine mosquito net restricting the in and out movement of aphids. The size of the plot net cage can be extended up to the coverage of complete test genotypes which can also be partitioned with a sheet of same material. For multi-choice natural infestation, the test genotypes were kept open to natural

Table 1 — Aphid population, damage and resistance indices to categorize rapeseed-mustard for resistance to *Lipaphis erysimi*

Aphid Population Index (API)	Aphid Damage Index (ADI)	Aphid Resistance Index (ARI)	Resistance Category
1 = No or less than 20 aphids on the inflorescences of test plants	1 = Normal plant growth, no symptoms of injury, no curling or yellowing of leaves	0.1-1.0 (API+ADI/2)	0.0-1.0 = Resistant
2 = upto 25% inflorescences have 21-100 aphids on the test plants	2 = Average plant growth, curling and yellowing of few leaves, flowering and fruiting	1.1-2.0 (API+ADI/2)	1.1-2.0 = Moderately resistant
3 = upto 50% of inflorescences have 101-250 aphids across test plants	3 = Poor plant growth, curling and yellowing of leaves on some branches, drying of few flowers and poor pod setting	2.1-3.0 (API+ADI/2)	2.1-2.5 = Tolerant
4 = upto 75% inflorescences have 251-500 aphids across test plants	4 = Stunted plant growth, heavy curling and yellowing of leaves all through the plant, drying and curling of almost half the inflorescence with poor flowering and rare pod setting	3.1-4.0 (API+ADI/2)	2.6-3.5 = Susceptible
5 = 100% of inflorescences have more than 500 aphids across test plants	5 = Severe stunting and ragged plant appearance, yellowing and curling of almost all the leaves, complete drying of inflorescence without any flower and immature drying of pods if any	4.1-5.0 (API+ADI/2)	3.6-5.0 = Highly susceptible

infestation by *L. erysimi* (Fig. 1E). Ten randomly selected plants of each test genotype from middle two rows in each replication were inoculated using aforesaid twig method of artificial infestation at bud initiation stage for multi-choice cage screening technique, while for natural multi-choice test the genotypes received natural *L. erysimi* infestation. Before inoculation it was ensured that there were no aphid and other non-target insects including natural enemies on the test plants.

Observations recorded

The observations were recorded on number of aphids, aphid population index (API), aphid damage index (ADI), and aphid resistance index (ARI) for all the test screening techniques. The observations on API were recorded after 21 days of inoculation, while ADI at completion of pod formation as per the index methods described in Table 1. The ARI were determined based on API and ADI values, and ultimately derived at resistance category (Table 1). In case of no-choice screening techniques, total numbers of aphids were also counted on the inoculated twig in twig cage technique and on the inoculated plants in case of whole plant cage technique after 21 days of inoculation. These total numbers of aphids per plant or twig for respective cage techniques were used to calculate aphid multiplication rate (AMR) = (Total number of aphids/Number of aphids released) × 100.

Data analysis

The data were subjected to analysis of variance (ANOVA) using the statistical software SAS® version 9.2. The data on different aphid screening techniques were analyzed in a factorial design with genotypes as the main treatment, and the screening techniques and seasons as sub-treatments. The significance of differences between genotypes, screening techniques, seasons, and their interactions were measured by F test, and the treatment means were compared using the least significant difference (LSD) at $P = 0.05$.

Results

Standardization of artificial infestation and screening techniques

Our initial efforts to standardize susceptible stage and appropriate plant site to infest aphids revealed that the artificial infestation of *L. erysimi* at bud formation in mustard crop is most appropriate stage and site to enable aphids to hide and establish. Considering the ease of inoculation and establishment of aphids, inoculation with the *L. erysimi* infested mustard twig pieces having ~20 mixed stage aphids pinned with bell pins on the top third branch was found effective and satisfactory, thus used for artificial infestation. Caging of mustard plants with *L. erysimi* protected them from natural enemies and restricted their migration away from the plants. Among the screening techniques, cage techniques for no-choice (twig cage and whole plant cage) and multi-choice (plot cage) conditions were found effective to screen rapeseed-mustard genotypes for resistance to *L. erysimi* as compared to natural infestation conditions. Many a times, the high *L. erysimi* population at flowering and pod setting doesn't translate into considerable plant damage and yield loss, on the other hand low *L. erysimi* population some time translates into severe plant damage and yield loss. Looking at these situations, certain damage evaluation procedures were devised and translated into various indices to differentiate test rapeseed-mustard genotypes into different *L. erysimi* resistance categories (Table 1).

***Lipaphis erysimi* resistance indices under no-choice screening techniques**

There were significant differences in aphid population index ($F_{5,70} = 61.39$; $P < 0.001$), aphid damage index ($F_{5,70} = 12.6$; $P < 0.001$), and aphid resistance index ($F_{5,70} = 44.61$; $P < 0.001$) between mustard genotypes, under different no-choice screening techniques (API: $F_{5,70} = 14.43$, $P = 0.017$; ADI: $F_{5,70} = 2.99$, P = 0.017; ARI: $F_{5,70} = 10.08$, $P < 0.001$), and across seasons (API: $F_{10,70} = 20.82$, $P < 0.001$; ADI: $F_{10,70} = 5.81$, $P < 0.001$; ARI: $F_{10,70} = 15.70$, $P < 0.001$) evaluated for *L. erysimi* resistance under artificial infestation conditions in the field (Table 2). Under twig cage technique, the aphid population index, aphid damage index and aphid resistance index were significantly higher in Heera as compared to other test genotypes (Table 2). Further, PDZM 31 and PM 21, and PM 30, Pusa Bold and Pusa Vijay were on par in aphid population index and aphid resistance index; while PM 21, PM 30, Pusa Bold and Pusa Vijay were on par in aphid damage index (Table 2). However, aphid damage index in Heera, PDZM 31, PM 30 and Pusa Bold; and aphid population index and aphid resistance index among Heera and PDZM 31, PM 30 and Pusa Bold, and PM 21 and Pusa Vijay were significantly on par under whole plant cage technique (Table 2). The *L. erysimi* damage parameters *viz.*, aphid population index ($F_{2,70} = 85.89$; $P < 0.001$), aphid damage index

Table 2 — Evaluation of mustard genotypes for resistance to aphid, *Lipaphis erysimi* under no-choice artificial infestation in (A) twig and (B) plant cages; and multi-choice in (C) plot cage artificial infestation and (D) natural infestation conditions in the field

Genotype	Screening techniques															
	No-choice								Multi-choice							
	2013		2014		2015		Mean		2013		2014		2015		Mean	
	A	B	A	B	A	B	A	B	C	D	C	D	C	D	C	D
Aphid Population Index																
Heera	4.40	3.40	4.81	3.81	4.68	4.43	4.63	3.88	2.03	3.00	3.89	4.68	4.71	4.64	3.54	4.11
PDZM 31	3.97	4.10	2.15	3.30	2.82	3.78	2.98	3.73	2.37	2.60	4.10	2.70	2.50	1.35	2.99	2.22
PM 21	2.97	2.67	3.93	1.91	1.98	2.61	2.96	2.39	2.33	2.60	2.25	4.47	1.40	1.86	1.99	2.98
PM 30	3.67	4.23	1.26	3.78	2.05	1.19	2.33	3.07	2.57	2.57	2.56	1.73	1.01	1.00	2.05	1.77
Pusa Bold	3.47	3.20	2.27	3.03	1.60	2.57	2.44	2.94	2.37	2.77	1.48	3.44	1.20	1.20	1.68	2.47
Pusa Vijay	3.67	4.30	1.33	1.44	2.37	1.41	2.45	2.38	2.37	3.27	1.21	2.54	1.63	1.71	1.74	2.51
Mean	3.69	3.65	2.62	2.88	2.58	2.66	-	-	2.34	2.80	2.58	3.26	2.07	1.96	-	-
Aphid Damage Index																
Heera	4.67	3.67	4.70	4.10	3.37	3.43	4.24	3.73	2.03	3.00	3.93	4.63	4.20	4.30	3.39	3.98
PDZM 31	4.03	4.27	4.00	4.60	2.27	2.27	3.43	3.71	2.37	2.60	4.07	4.27	1.77	1.20	2.73	2.69
PM 21	3.00	3.00	4.63	4.17	1.50	1.73	3.04	2.97	2.37	2.63	3.60	4.57	1.63	1.33	2.53	2.84
PM 30	3.73	4.23	2.90	4.63	1.73	1.43	2.79	3.43	2.57	2.57	3.40	3.53	1.27	1.10	2.41	2.40
Pusa Bold	3.37	3.57	3.60	3.97	1.70	1.80	2.89	3.11	2.37	2.77	3.10	4.57	1.23	1.30	2.23	2.88
Pusa Vijay	3.87	4.50	3.47	2.87	1.87	1.30	3.07	2.89	2.37	3.27	3.47	3.93	1.57	1.77	2.47	2.99
Mean	3.78	3.87	3.88	4.06	2.07	1.99	-	-	2.34	2.81	3.59	4.25	1.94	1.83	-	-
Aphid Resistance Index																
Heera	4.53	3.53	4.75	3.96	4.03	3.93	4.44	3.81	2.03	3.00	3.91	4.66	4.45	4.47	3.47	4.04
PDZM 31	4.00	4.18	3.07	3.95	2.54	3.03	3.21	3.72	2.37	2.60	4.08	3.48	2.13	1.27	2.86	2.45
PM 21	2.98	2.83	4.28	3.04	1.74	2.17	3.00	2.68	2.35	2.62	2.92	4.52	1.52	1.60	2.26	2.91
PM 30	3.70	4.23	2.08	4.21	1.89	1.31	2.56	3.25	2.57	2.57	2.98	2.63	1.14	1.05	2.23	2.08
Pusa Bold	3.42	3.38	2.93	3.50	1.65	2.19	2.67	3.02	2.37	2.77	2.29	4.00	1.22	1.25	1.96	2.67
Pusa Vijay	3.77	4.40	2.40	2.15	2.12	1.35	2.76	2.64	2.37	3.27	2.34	3.24	1.60	1.74	2.10	2.75
Mean	3.73	3.76	3.25	3.47	2.33	2.33	-	-	2.34	2.80	3.09	3.76	2.01	1.90	-	-

LSD for comparing	Aphid Damage Index	Aphid Population Index	Aphid Resistance Index	Aphid Damage Index	Aphid Population Index	Aphid Resistance Index
Technique (T)	0.19	0.14	0.13	0.13	0.11	0.11
Genotype (G)	0.33	0.25	0.24	0.23	0.19	0.19
Season (S)	0.23	0.17	0.16	0.16	0.13	0.13
T × G	0.47	0.35	0.33	0.33	0.27	0.26
T × S	0.33	0.25	0.23	0.23	0.19	0.19
G × S	0.57	0.43	0.40	0.4	0.33	0.32
T × G × S	0.81	0.60	0.57	0.57	0.47	0.46

($F_{2,70}$ = 170.13; P <0.001), and aphid resistance index ($F_{2,70}$ = 160.48; P <0.001) also varied significantly between test genotypes during different crop seasons (Table 2). The aphid population index ($F_{1,70}$ = 1.95; P = 0.166), aphid damage index ($F_{1,70}$ = 0.43; P = 0.512), and aphid resistance index ($F_{1,70}$ = 1.47; P = 0.229) under test no-choice screening techniques, and the techniques × season interactions (API: $F_{2,70}$ = 1.45, P = 0.242; ADI: $F_{2,70}$ = 0.60, P = 0.552; ARI: $F_{2,70}$ = 1.00, P = 0.373) were found statistically non-significant, indicating season-neutral efficacy of these no-choice screening techniques to evaluate mustard genotypes for resistance to *L. erysimi* under field conditions. The interactions between no-choice screening techniques × genotypes × seasons also showed significant variability in aphid population index ($F_{10,70}$ = 13.46; P <0.001), aphid damage index ($F_{10,70}$ = 2.40; P = 0.016), and aphid resistance index ($F_{10,70}$ = 8.58; P <0.001) between mustard genotypes for *L. erysimi* resistance under artificial infestation conditions in the field (Table 2). Furthermore, the twig cage technique revealed maximum variability

among mustard genotypes for aphid population index (2.33 to 4.63 *vs*. 2.38 to 3.88; Table 2), aphid damage index (2.79 to 4.24 *vs*. 2.89 to 3.73; Table 2), and aphid resistance index (2.67 to 4.44 *vs*. 2.64 to 3.81; Table 2) as compared to plant cage technique. These findings indicate that the twig cage artificial screening technique is more efficient in differentiating mustard genotypes for slightest variation in levels of tolerance/ susceptibility to *L*. *erysimi*.

Reproduction of *L. erysimi* under no-choice screening techniques

There were significant differences between mustard genotypes for aphid population and rate of aphid multiplication ($F_{5,70}$ = 1138.77; P <0.001), under different no-choice screening techniques ($F_{5,70}$ = 68.02, P <0.001), and across seasons ($F_{10,70}$ = 80.54, P <0.001) evaluated for *L*. *erysimi* resistance under artificial infestation conditions in the field (Table 3). The numbers of *L*. *erysimi* and their multiplication rate under both twig cage and plant cage techniques were significantly higher on Heera, being lower on Pusa Vijay as compared to other genotypes (Table 3). Aphid population and aphid multiplication rates on PM 30 and Pusa Bold were significantly at par under twig cage technique, while under plant cage technique these were on par with each other on PM 21 and PM 30 (Table 3). The numbers of *L*. *erysimi* and their multiplication rate during different crop seasons ($F_{2,70}$ = 379.29; P <0.001) under test no-choice screening techniques ($F_{1,70}$ = 4.05; P = 0.0.048), and for techniques × season interactions ($F_{2,70}$ = 45.29, P <0.001) varied significantly between test mustard genotypes (Table 3). These results indicate significant effect of screening technique and cropping season on the rate of *L*. *erysimi* multiplication on different mustard genotypes even under artificial infestation conditions in the field. The interactions between screening techniques × genotypes × seasons also showed significant variability in number of *L*. *erysimi* and multiplication rate ($F_{10,70}$ = 230.82; P <0.001) between mustard genotypes under artificial infestation conditions in the field (Table 3). Highly variable rate

Table 3 — Multiplication of aphid, *Lipaphis erysimi* on different mustard genotypes under no-choice screening techniques under field conditions

Genotype	Screening Technique							
	Twig cage				Plant cage			
	2013	2014	2015	Mean	2013	2014	2015	Mean
Aphid Population (No. of aphids)								
Heera	1742	937	1166	1282	1143	1218	2275	1545
PDZM 31	644	563	1230	812	990	757	499	749
PM 21	1178	397	674	749	572	522	280	458
PM 30	379	410	768	519	1135	151	191	493
Pusa Bold	680	319	445	481	910	514	241	555
Pusa Vijay	399	473	362	411	432	281	327	347
Mean	837	517	774		1028	780	833	
Aphid Multiplication Rate (× times)								
Heera	87.1	46.8	58.3	64.1	57.2	60.9	113.8	77.3
PDZM 31	32.2	28.2	61.5	40.6	49.5	37.8	25.0	37.4
PM 21	58.9	19.8	33.7	37.5	28.6	26.1	14.0	22.9
PM 30	19.0	20.5	38.4	26.0	56.8	7.6	9.6	24.6
Pusa Bold	34.0	16.0	22.3	24.1	45.5	25.7	12.0	27.8
Pusa Vijay	19.9	23.7	18.1	20.6	21.6	14.1	16.3	17.3
Mean	41.8	25.8	38.7		43.2	28.7	31.8	
LSD (P = 0.05) for comparing	Aphid Population (No. of aphids)				Aphid Multiplication Rate (x times)			
Technique (T)	18.04				0.90			
Genotype (G)	31.25				1.56			
Season (S)	22.1				1.11			
T × G	44.19				2.21			
T × S	31.25				1.56			
G × S	54.12				2.71			
T × G × S	76.54				3.83			

of *L. erysimi* multiplication under plant cage (17.3 to 77.3) as compared to twig cage (20.6 to 64.1) across mustard genotypes (Table 3), further indicate that the twig cage technique is proficient and précised for artificial screening of mustard genotypes for resistance to *L. erysimi*.

L. erysimi resistance indices under multi-choice screening techniques

Screening of mustard genotypes under multi-choice conditions revealed that the aphid population index ($F_{5,70}$ = 108.19; P <0.001), aphid damage index ($F_{5,70}$ = 30.32; P <0.001), and aphid resistance index ($F_{5,70}$ = 75.04; P <0.001) varied significantly among genotypes, screening techniques (API: $F_{5,70}$ = 27.30, P = <0.001; ADI: $F_{5,70}$ = 3.41, P = 0.017; ARI: $F_{5,70}$ = 13.57, P <0.001), and seasons (API: $F_{10,70}$ = 44.03, P <0.001; ADI: $F_{10,70}$ = 21.44, P <0.001; ARI: $F_{10,70}$ = 37.26, P <0.001) for *L. erysimi* resistance under field conditions (Table 2). The aphid population index, aphid damage index and aphid resistance index were significantly higher in Heera followed by PDZM 31 as compared to other test genotypes; being significantly on par among PM 21, PM 30, Pusa Bold and Pusa Vijay under plot cage technique (Table 2). However, these resistance parameters were highly variable across genotypes and seasons under natural infestation conditions, except Heera which was found consistently susceptible to *L. erysimi* (Table 2). The *L. erysimi* damage parameters *viz.*, aphid population index ($F_{2,70}$ = 91.73; P <0.001), aphid damage index ($F_{2,70}$ = 317.76; P <0.001), and aphid resistance index ($F_{2,70}$ = 249.96; P <0.001) varied significantly for test mustard genotypes across seasons. The aphid population index ($F_{1,70}$ = 38.72; P <0.001), aphid damage index ($F_{1,70}$ = 25.02; P <0.001), and aphid resistance index ($F_{1,70}$ = 39.51; P <0.001) under multi-choice screening techniques, and the techniques × season interactions (API: $F_{2,70}$ = 18.68, P <0.001; ADI: $F_{2,70}$ = 11.79, P <0.001; ARI: $F_{2,70}$ = 18.84, P <0.001) were also found significant, indicating seasonal effect on the efficacy of these multi-choice screening techniques to evaluate mustard genotypes for resistance to *L. erysimi* under field conditions. The aphid population index, aphid damage index and aphid resistance index across genotypes during different seasons were significantly higher during 2014 followed by 2013 and 2015 under both the multi-choice screening techniques. The interactions between multi-choice screening techniques × genotypes × seasons also showed significant variability in aphid population index ($F_{10,70}$ = 10.84; P <0.001), aphid damage index ($F_{10,70}$ = 1.32; P = 0.016), and aphid resistance index ($F_{10,70}$ = 4.90; P <0.001) between mustard genotypes for *L. erysimi* resistance (Table 2). Furthermore, multi-choice natural infestation conditions although revealed maximum variability among test mustard genotypes for aphid population index (1.68 to 3.54 *vs*. 1.77 to 4.11; Table 2), aphid damage index (2.23 to 3.39 *vs*. 2.40 to 3.98; Table 2), and aphid resistance index (1.96 to 3.47 *vs*. 2.08 to 4.04; Table 2), but unregulated, inconsistent and higher as compared to those under plot cage artificial infestation conditions. For example, the aphid population index, aphid damage index and aphid resistance index in Heera, PM 21, Pusa Bold and Pusa Vijay were significantly higher under natural infestation conditions, while the aphid population index in PDZM 31 and PM 30 was lower under multi-choice plot cage technique. Further, in spite of being higher aphid population index, aphid damage index was on par in PDZM 31 and PM 30 under both the multi-choice screening conditions. These studies thus indicate that the plot cage artificial screening technique is more appropriate to differentiate mustard genotypes for resistance to *L. erysimi* under controlled release than under natural infestation conditions.

Resistance categorization of test mustard genotypes against _L. erysimi_

The evaluation of double low erucic acid and total glucosinolate, single low erucic acid, and conventional varieties with high erucic acid and total glucosinolate mustard genotypes for *L. erysimi*

Table 4 — Categorization of test mustard genotypes for *Lipaphis erysimi* using aphid population and damage indices

Screening techniques	Mustard genotypes					
	Heera	PDZM 31	PM 21	PM 30	Pusa Bold	Pusa Vijay
Twig cage	Highly susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Plant cage	Highly susceptible	Highly susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Plot cage	Susceptible	Susceptible	Tolerant	Tolerant	Moderately resistant	Tolerant
Across cage techniques	Highly susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Tolerant
Natural infestation	Highly susceptible	Tolerant	Susceptible	Tolerant	Susceptible	Susceptible

resistance showed that the aphid population index (Fig. 2A), aphid damage index (Fig. 2B), aphid resistance index (Fig. 2C), and aphid multiplication rate (except under multi-choice cage and natural infestation conditions for this parameter; Fig. 2D) were significantly higher in double low erucic acid and total glucosinolate genotypes across the test screening techniques as compared to other group of genotypes (Fig. 2). There was no significant difference for aphid damage index (Fig. 2B) and aphid multiplication rate (Fig. 2D) on single low erucic acid, and conventional varieties with high erucic acid and total glucosinolate group of mustard genotypes under either of the screening techniques, except under twig cage technique. Genotype Heera was found susceptible to *L. erysimi* under plot cage technique, while highly susceptible under no-choice screening techniques and natural infestation (Table 4). Genotype PDZM 31 was found consistently susceptible to highly susceptible to *L. erysimi* under no-choice and multi-choice artificial infestation conditions, while it showed tolerant reaction under natural infestation conditions, could be due to migration to more preferred susceptible staged plants. Genotypes PM 21, Pusa Bold and Pusa Vijay were found susceptible under both no-choice cage screening techniques as well as under natural infestation conditions, while these genotypes showed tolerant to moderately resistant reaction under plot cage technique, might be due to lateral spread of inoculated aphids to adjacent non-inoculated plants (Table 4). Genotype PM 30 was found tolerant under multi-choice plot cage and natural infestation conditions, however susceptible under no-choice cage screening techniques, indicating that the no-choice cage techniques are more reliable to identify resistant genotypes as compared to multi-choice natural or artificial infestation techniques. Furthermore, both the no-choice cage screening techniques were although equally effective and consistent, the ease of handling, cost of the cage (as twig cage costs half the plant cage), and plant growth under natural microclimatic conditions (except the infested twigs) are some of the important factors which sounds the twig cage technique more précised and appropriate for artificial screening of rapeseed-mustard for resistance to *L. erysimi*.

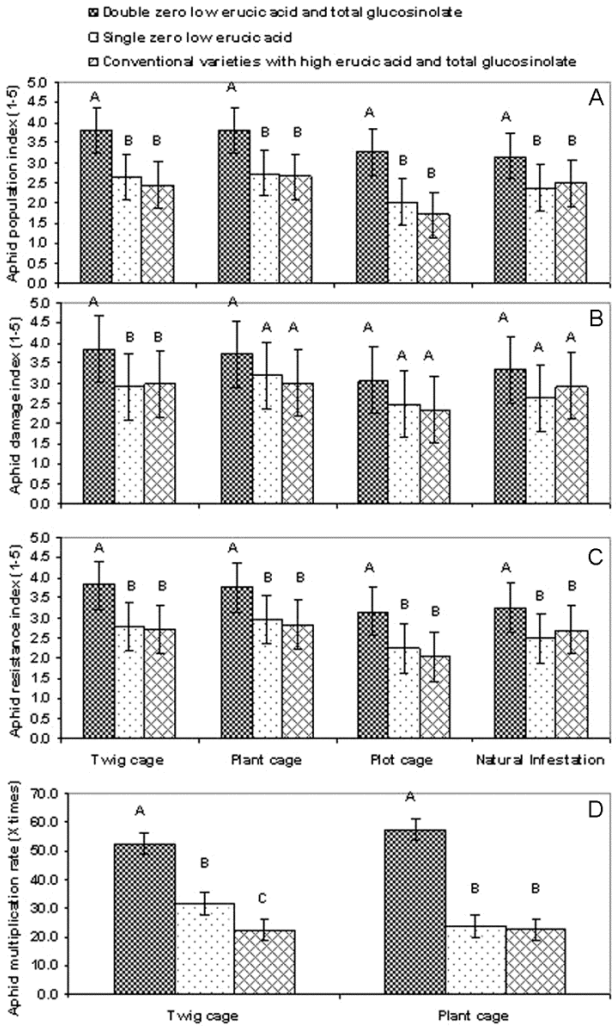

Fig. 2 — (A) Aphid population index; (B) aphid damage index; (C) aphid resistance index; and (D) aphid multiplication rate in diverse groups of mustard genotypes for resistance to aphid, *Lipaphis erysimi* under different screening conditions in the field.

Discussion

The population and damage by aphid, *L. erysimi* in rapeseed-mustard varies across seasons and regions, and is difficult to identify genotypes with resistance to this pest under natural infestation conditions. Several techniques have been in use for infestation and evaluation of the test material in the field for many crops[26-28]. However, due to lack of such dependable technique to screen rapeseed-mustard for resistance against *L. erysimi*, aphid resistance breeding program in this crop has not been rewarding. The amount of food available, multiplication rate and the insect density influence expression of resistance, thus optimum and uniform level of infestation is emphasized to get maximum differences between the resistant and susceptible genotypes[29]. Standardization of techniques to inoculate the rapeseed-mustard plants at the susceptible stage with uniform insect density and damage evaluation procedures is essential for success of

the insect resistance screening program. Present studies found that the artificial infestation of mustard genotypes with *L. erysimi* at bud formation stage is most appropriate for the establishment of aphids at the inoculation site. Further, the inoculation with *L. erysimi* infested mustard twig pieces having around 20 mixed stage aphids pinned with bell pins on the top third branch near inflorescence was found effective and satisfactory for artificial infestation. Certain resistance evaluation procedures encompassing *L. erysimi* population and damage levels were also devised to differentiate test mustard genotypes into different resistance categories.

The screening for insect resistance can be carried out under multi-choice, dual-choice, or no-choice conditions using appropriate artificial infestation and cage techniques. The choice or no-choice insect resistance screening techniques have earlier been reported to be successful in identifying sources of resistance to sucking pests in several crops[30-34]. Immense efforts have also been made in the past to identify sources of resistance to aphids in *Brassica* species[21-23,35-37], which also indicate that the resistance in the primary gene pool of rapeseed-mustard to *L. erysimi* are rare bearing low to moderate levels of resistance. But most of these studies have been under natural infestation conditions, wherein several factors interplay to counterfeit to identify the resistant/ tolerant genotypes. Sucking insects particularly the aphids have specialized feeding habit and ingest enormous phloem fluid to fulfill the desired nutritional requirement. The aphid bioassay techniques like detached plant part bioassay[38] and pot cage screening technique[24] have also been used for evaluation of rapeseed-mustard for aphid resistance. The detached plant parts like leaves, inflorescence, pods, or fruits are inefficient to cater the nutritional requirement of aphids as do the naturally growing plants. The bioassays with attached *vs.* excised leaves of alfalfa for resistance to spotted alfalfa aphid, *Therioaphis maculata* (Buckton) although revealed significant differences in nymphal survival but it was greater on excised leaves than on intact leaves, suggesting that the excised leaves underestimate the levels of resistance in the test plants[39]. The bioassays with excised leaves have also been reported associated with induced resistance, and is not representative of the plant organ[40]. Pot cage bioassays are although useful for evaluation of mustard for aphid resistance[24], the abiotic and edaphic plant growing conditions, physiological and nutritional deviation from naturally grown host plants, and their limitation to use for large number of genotypes restrict the scope of this technique in final decision making on the test plant's resistance/susceptibility reaction.

Caging the mustard plants with *L. erysimi* is dependable method of screening for insect resistance under field conditions, wherein considerable control is exercised to maintain uniform insect pressure by artificial inoculation at the same plant phenological stage. It also protects the aphids from natural enemies and restricts their movement away from the test plants. The cages can be designed to cover the whole plants or only the plant parts that are more prone to insect damage. Present studies revealed significant differences in aphid population index, aphid damage index, and aphid resistance index between mustard genotypes, under no-choice cage screening techniques and across seasons for *L. erysimi* resistance under artificial infestation conditions in the field. The non-significant differences for various aphid resistance indices under no-choice screening techniques and the techniques × season interactions indicate season-neutral efficacy of these techniques to evaluate mustard genotypes for resistance to *L. erysimi*. However, significant variation in *L. erysimi* damage parameters for techniques × season interactions indicated seasonal effect on the efficacy of multi-choice screening techniques to evaluate mustard genotypes for resistance to *L. erysimi* under field conditions. Furthermore, evaluation under multi-choice natural infestation conditions although revealed maximum variability in aphid resistance parameters among the test mustard genotypes, but it was unregulated, inconsistent and higher damage levels as compared to those under plot cage artificial infestation conditions. Efforts have also been made earlier to assess the resistance in several oleferous rapeseed-mustard species by observing aphid setting and alate production in *L. erysimi* after inoculation via infested inflorescence under field conditions[41]. These studies observed significant differences in aphid settling and alate formation on the test rapeseed mustard species, however no further reference has been made on standardization, validation or use of infested inflorescence method of aphid inoculation. Thus, plot cage artificial screening technique for multi-choice assays is more appropriate as compared to natural infestation conditions.

The numbers and multiplication rate of *L. erysimi* were significantly higher on Heera, being lower on Pusa Vijay as compared to other genotypes under both twig cage and plant cage techniques. However, the numbers and multiplication rate of *L. erysimi* on

PM 30 and Pusa Bold under twig cage technique and on PM 21 and PM 30 under plant cage technique were statistically on par with each other. These variations indicate significant effect of screening technique and cropping season on the rate of *L. erysimi* multiplication on different mustard genotypes even under artificial infestation conditions in the field. Genotype PDZM 31 was found consistently susceptible to highly susceptible to *L. erysimi* under no-choice and multi-choice artificial infestation conditions, while it showed tolerant reaction under natural infestation conditions. However, tolerant reaction of PM 30 under multi-choice plot cage and natural infestation conditions and susceptible under no-choice cage conditions, indicate that the no-choice cage screening techniques are more reliable than the multi-choice ones to identify *L. erysimi* resistant mustard genotypes. Furthermore, maximum variability among test mustard genotypes for all the aphid damage parameters under twig cage technique as compared to plant cage technique, suggest that the twig cage artificial screening technique is more appropriate to differentiate mustard genotypes at slightest variation in levels of tolerance/ susceptibility to *L. erysimi*. The of twig cage technique also efficiently differentiated the double low erucic acid and total glucosinolate, single low erucic acid, and conventional varieties with high erucic acid and total glucosinolate groups of mustard genotypes for *L. erysimi* resistance. The high variability in multiplication rate of aphids across test mustard genotypes and difficulty in scouting of aphids throughout the test plant under plant cage technique as compared to twig cage technique further endorse the appropriateness and precision of twig cage technique for artificial screening of mustard for resistance to *L. erysimi*. The no-choice cage techniques were although equally effective, but ease of handling, cost of the cage and plant growth under natural microclimatic conditions (except only the aphid inoculated twigs) are some additional indicators which also support that the twig cage technique is more précised, realistic, economical, and efficient for artificial screening of mustard for resistance to *L. erysimi*.

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