Factors involved in enhancing host susceptibility towards aphid clonal propagation on leaf foliage of *Arabidopsis*

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The present study identified factors that enhanced host susceptibility towards *Myzus persicae*'s clonal proliferation in the model plant, *Arabidopsis thaliana*. A particular aphid inoculum, i.e. five aphids release per plant among three inoculums (1, 5 and 10 aphids per plant) showed enhanced susceptibility towards aphid clonal propagation in 21-day-old *Arabidopsis* leaf foliage. Five aphid number was common among the 28, 35, 42, 49-day-old *Arabidopsis* leaf foliage except 42-day-old *Arabidopsis*. Prior aphid herbivory enhanced host susceptibility in *Arabidopsis*. The aphid inoculum at 6 am showed enhanced host susceptibility in comparison to 12 noon and 6 pm. The enhanced susceptibility on leaf foliage was realized in the presence of flower stalk. Aphid preferred to proliferate significantly on the flower stalk as compared to leaf foliage. Within leaf, aphid colonized more in mid-rib region as compared to minor vein area in the mature leaf. Pro^{PAD4}::GUS and Pro^{ADF3}::GUS showed poor expression in mid-rib region in aphid-challenged leaf foliage. The aphid feeding based primed vascular sap showed degraded peptide bond, a possible reason for favouring enhanced aphid clonal proliferation in the primed *Arabidopsis* leaf foliage. Results showed that the enhancing of host susceptibility in *Arabidopsis* to *Myzus persicae* is influenced by quorum number of aphid's inoculum, prior aphid feeding and circadian rhythms. Differential spatial resistance within whole plant and within leaf was also recorded. The enhanced host susceptibility was also correlated with microbiota enrichment in aphid-herbivore leaf vasculature sap as well as aphid body including aphid apical part containing salivary gland.

Keywords: *Arabidopsis thaliana*, aphid inoculums, enhanced host susceptibility, spatial resistance, timing of aphid inoculums.

APHIDS are recognized as pests globally due to significant yield losses to agricultural and horticultural crops[1]. Amongst the 4000 aphid species documented, approximately 250 are recognized as pests[2,3]. Aphids are also classified into generalists and specialists based on host range. A generalist aphid, such as the green peach aphid (GPA; *Myzus persicae*) is able to proliferate on a wide array of plant species and is known as polyphagous[2,4]. On the other hand, specialist aphid enables to proliferate clonally by tapping the phloem sap on a limited number of related plants species[4]. For example, mustard aphid (*Lipaphis erysimi*) colonizes only on *Cruciferous* plants[2]. A number of factors from plant surface, epi-vascular region and vascular region could modulate host susceptibility[5–8]. Aphid resistance may be influenced by leaf surface-specific factors including leaf colour, emitted volatile organic compounds and leaf surface components, such as epicuticular waxes or trichomes[9–11]. The presence or expression of mannose-binding lectin in the phloem tissue could confer resistance to aphid clonal proliferation[12–14]. Specialized mouthparts of aphids known as stylets, were explored to navigate towards nutrient-enriched phloem sap[5–8,15]. The aphid stylet-based feeding transmitted plant pathogenic viruses on the host plant[8,16–18] which could potentially reduce crop yields[19]. Aphid stylet navigation ruptured all layers of mesophyll cell, and finally penetrated into SE element cell[5–7]. During stylet navigation, every and each cell sap was tested by aphid and based on sap quality, aphid stylet navigation is oriented towards SE element cell[5–7]. Aphid also secreted two types of saliva, watery and sheath type, both in the apoplast and into probed cells along the stylet-pathway[19,20]. In compatible plant host (susceptible host), aphid establishes successful salivation with the SE element cell reversing the SE element occlusion[5–7]. After the establishment of successful contact with the SE element, aphid tapped a huge volume of sugar enriched SE element sap which fulfilled the aphid nutrition resulting in rapid clonal proliferation in a suitable environment[20,21].

The polyphagous GPA *Myzus persicae* (Sülzer) (Hemiptera: Aphididae) has an exceptionally wide host range covering more than 50 families of plants[1,2]. A number of factors like carbohydrate metabolism, premature senescence, plant hormones, resistance genes, non-protein amino acids, lipid-like 9-LOX-derived oxylipins, fatty acid desaturases, trehalose metabolism modulated the host resistance[22]. For evaluating host resistance, it is mandatory to follow *in-planta* bioassay for evaluating mutant, or transgenic line.

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From the literature, it was found that a variable number of aphid inoculums ranges from one to twenty aphids release per plant which was considered to evaluate the host resistance[6,7,23–28]. Moreover, the timing of aphid release was not mentioned clearly[5,6,13,22,23,26–28]. The age of the host plant was mentioned but the host plant development may vary based on an environmental condition which also varied from laboratory to laboratory. In the full-grown plant, the place of aphid inoculum was mentioned but spatial resistance might exist[5,6,13,22,23,26–28]. In addition, the prior aphid infestation during plant development in the laboratory condition modulated resistance[5,6,11,13,22,23,26,27]. The present study identified some parameters like initial aphid inoculums, timing of aphid inoculum and prior aphid infestation history which modulated the host resistance. In addition, the developmental stage of plant, presence of flower stalk, spatial resistance within the plant and spatial resistance within leaf blade were identified as modulating factors for aphid clonal proliferation. The knowledge will be fine-tuned to plant–aphid interaction biology for addressing host resistance.

Materials and method

Definition of TAP, TAPI and RTAP

Total aphid proliferation (TAP) is defined as the total number of proliferated aphids including initial aphid inoculum. The time of aphid proliferation as compared to initial (TAPI) was the ratio of total aphid number at a particular time point divided by initial aphid release at 0 h time point. The real times aphid proliferation (RTAP) was the ratio of total aphid proliferation at any time point divided by previous time point's aphid number. The RTAP at 0 h was assumed to be one. The significant RTAP indicates that the value of RTAP was significantly higher than one.

Arabidopsis growth

The model plant, *Arabidopsis thaliana* (Col-0) was used for the present study. The stock no. CS70000 was received from the *Arabidopsis* Information Resource (TAIR) for all experiments. The *Arabidopsis* seed bed was incubated for 2 days at 4°C for achieving uniform and enhanced germination. Subsequently *Arabidopsis* was cultured in plant growth room conditioned with 6000 LUX of light intensity, 14 h light condition per day and 70% of relative humidity.

Aphid culture

The *Myzus persicae* (kingdom: *Animalia*, rank: *Species*, higher classification: *Myzus*, family: Aphididae, order: Hemiptera) was used for the study. The square-shaped pot was filled with artificial soil, Soilrite (Keltech Energies Ltd, Bengaluru, India), which was used for Radish, Bombay Red Khashi Katta (Pooja Seeds) and *Brassica* (Pooja Seeds) plant in the environment conditioned with 6000 LUX of light intensity, 14 h light condition per day and 70% of relative humidity in plant growth chamber (PGC).

Arabidopsis bioassay

The *Arabidopsis* plant of 21-day-old was considered for *in-planta* bioassay with three doses, i.e. 1, 5 and 10 aphids release per plant. Aphid was released on leaf foliage of *Arabidopsis thaliana* (6 replications). The plant was selected at random from culturing tray and the proliferated aphid was monitored at different time points, i.e. 12, 36, 60, 84 and 108 h.

Different growth stages bioassay

Three doses such as 1, 5 and 10 aphids per plant were followed to identify the earliest and significant TAP and RTAP in different growth stages of 28, 35, 42 and 49-day-old *Arabidopsis* (6 replications). The 28-day old *Arabidopsis* had only leaf, no shoot initiation; 35-day-old *Arabidopsis* had a greater number of leaves as compared to 28-day-old plant. The 42-day-old *Arabidopsis* had short shoot initiation with flower only, not the matured silique. 49-day-old *Arabidopsis* had long shoot having at least one mature silique. Aphid was released in the evening (6 pm) on leaf foliage.

Prior aphid infested leaf foliage bioassay

Five aphids were released on each *Arabidopsis* plant (6 replications). After 24 h of incubation, all the aphids were removed. The 0 h gap means a new bioassay experiment was initiated after removing the previous aphid without any time gap and 24 h gap means new bioassay experiment was initiated after 24 h gap period from the removal of the previous aphids. The priming was done by *Myzus persicae* herbivory for 24 h on the leaf foliage. After 24 h, all aphids were removed.

Different time point bioassay

One adult aphid was released on the leaf foliage at 28-day-old *Arabidopsis* (6 replications) in different time points, i.e. 6 am, 12 noon and 6 pm. The aphid clonal propagation was monitored at different time points, i.e. 12 h, 36 h, 60 h, 84 h and 108 h from the time of aphid inoculum. One aphid was released instead of five to remove the dose-effect recorded from the previous experiment.

Bioassay at flower stalk (shoot)

One aphid was released into each *Arabidopsis* leaf foliage (6 replications) having flower stalk and without flower stalk but the aphid inoculum was positioned into the leaf foliage only. More importantly, the aphid proliferation was counted only in leaf foliage to evaluate any effect of the presence of flower stalk. One aphid was released instead of five to remove the dose-effect realized from the previous experiment.

Bioassay on leaf without shoot and vice versa

42-day-old *Arabidopsis* having shoot was considered for this experiment. The leaf was removed and the shoot (shoot without leaf) for bioassay was considered. Similarly, the shoot was removed and the leaf foliage was considered (leaf without shoot) for bioassay. Five aphids were released on either shoot or leaf (6 replications). The aphid clonal proliferation was monitored at different time points.

Spatial resistance assay

Five aphids were released on *Arabidopsis* leaf foliage without any restricted movement within the same plant. The aphid clonal propagation in both flower stalk portion and leaf foliage was monitored at 12 h, 36 h, 60 h, 84 h and 108 h. The same experiment was performed when aphid inoculum was released on flower stalk. The clonal propagation of aphid on shoot and leaf when aphid release was followed on leaf after removal of shoot (leaf without shoot) and shoot after removal of leaf (shoot without leaf). 42-day-old *Arabidopsis* having shoot was considered for this experiment. The leaf was removed and the shoot (shoot without leaf) for bioassay was considered. Similarly, shoot was removed and the leaf foliage (leaf without shoot) for bioassay was considered. Five aphids were released on either shoot or leaf (6 replications). The aphid clonal proliferation was monitored in different time points.

Aphid settlement in a leaf

Five aphids were released on a leaf blade and the aphid clonal proliferation up to 96 h was monitored. The aphid position was divided into two categories namely aphid physical orientation to mid-rib and outside mid-rib area in the same leaf blade.

Histochemical study

The GUS staining assay was done according to protocol described by Mondal *et al.*[7]. The aphid-challenged leaf was cut from the *Arabidopsis* foliage and quickly dipped into 2 ml of X-Gluc stain in 24-well plates. After dipping the aphid-challenged leaf in X-Gluc solution, it was immediately incubated under low pressure for 10 min to kill the aphid for achieving the stylet penetrated leaf for monitoring GUS expression at aphid feeding site. After incubation of low-pressure suction, the plates were incubated for 6 h at 37°C. After staining, sample was incubated in 70% ethanol for overnight. The snapshot was taken for documentation.

Absorbance signature

Five aphids were released on each *Arabidopsis* plant. After 24 h of incubation, all the aphids were removed. After removal of aphids, the aphid feeding based-primed plant was kept for another 24 h without aphid incubation. Leaf exudate was isolated from 48 h which was 24 h aphid incubation and 24 h gap period. Four leaves were collected from aphid-challenged *Arabidopsis* and incubated in 2 ml of 1 mM EDTA, pH 8.0 for 16 h overnight. 24 ml of 1 mM EDTA, pH 8.0 was filtered through 0.22 µm membrane and lyophilized to 0.2 ml. The 20 µl lyophilized leaf exudate was diluted in 2 ml of Millipore water. The absorbance signature was recorded from 190 to 800 nm of aphid-challenged leaf exudate.

Bradford assay

Five aphids were released on each *Arabidopsis* plant. After 48 h of incubation, all the aphids were removed. After removal of aphids, leaf exudate was isolated from aphid-challenged foliage. Four aphid-challenged leaves were incubated in 2 ml of 1 mM EDTA, pH 8.0 for 16 h overnight. 24 ml of 1 mM EDTA, pH 8.0 was filtered through 0.22 µm membrane and 200 µl was inoculated for Bradford assay to record the absorbance.

Microbiota titer assay

Ten individual aphids were collected from 1 aphid per plant leaf foliage and 5 aphids per plant leaf foliage at 36 h. The aphid apical part (AAP) containing salivary gland and stylet were dissected under microscope. The AAP were homogenized in 1 ml of 1 mM EDTA, pH 8.0. The 50 µl of homogenized suspension was added to 1.6 ml of LB liquid media. The 50 µl EDTA, pH 8.0 was added to LB for blank during absorption at 595 nm.

Single aphid microbiota titer

Only one aphid was considered for this study. One aphid was collected from lower and higher dose of aphid-challenged leaf foliage (6 replications). The collected aphid was just dropped into 1.6 ml of $0.125 \times$ LB and absorbance was measured on the next day at 595 nm.

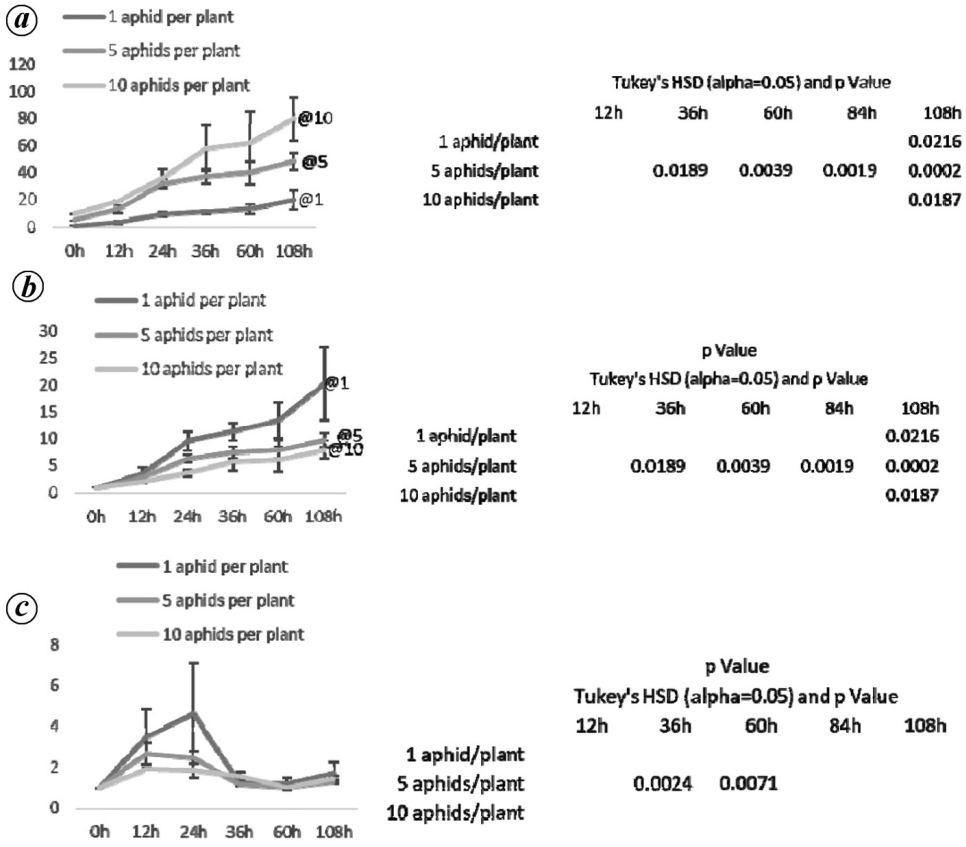

Figure 1 ***a–c.*** Initial aphid inoculum modulated host susceptibility towards aphid clonal propagation in the model plant, *Arabidopsis thaliana*.

Microbiota titer in vasculature sap

One and five aphids were released on *Arabidopsis* leaf foliage and incubated for 36 h. After 36 h, aphid-challenged leaf was considered for exudate isolation for 4 h in 1 mM EDTA, pH 8.0 in the day time. One aphid-challenged leaf was considered for exudate isolation in 1.6 ml of 1 mM EDTA, pH 8.0. 50 µl exudate was inoculated into 1.6 ml of $0.125 \times$ LB and incubated for overnight at 37°C, 120 rpm. As a control, 50 µm of 1 mM EDTA, pH 8.0 was inoculated into LB liquid media which was used as blank in recording absorbance the data in spectrophotometer.

Software used

One-way ANOVA and Tukey's HSD calculator was used for calculation (https://www.icalcu.com/stat/anova-tukey-hsd-calculator.html) to calculate *P* values at 0.05% level of significance to see any significant difference among the different time points for a particular aphid inoculum. The MedCalc statistical software (https://www.medcalc.org/calc/comparison_of_means.php) was also utilized to calculate the difference between the observed means in two independent samples.

Result and discussion

The experiment was initiated with three different doses of aphid inoculums such as 1, 5 and 10 aphids release onto the leaf foliage in each plant. It was expected that the earliest and significant aphid proliferation as compared to initial aphid release will be the same in all three doses. However, only five aphid releases showed the earliest and significant aphid clonal propagation as compared to the initial aphid inoculum at 36 h (Figure 1 *a* and *b*). The experiment was repeated three times with similar result. This result conceptualized the idea of a particular number of aphid inoculum enhanced the host susceptibility similar to 'quorum' concept established in the bacterial system when a particular gene is expressed based on bacterial density. The RTAP was also significantly higher in 5-aphids inoculum (Figure 1 *c*).

It was evident from the previous result that a particular aphid inoculum showed enhanced host susceptibility towards aphid clonal propagation on the 21-day-old *Arabidopsis* foliage. The next experiment was conceptualized to validate 'quorum' number of aphid inoculum that enhanced host susceptibility in different ages such as 28, 35, 42 and 48-days *Arabidopsis* plant from 1, 5 and 10 aphids release in a single plant. The 28-day-old *Arabidopsis* showed

(a)

Arabidopsis thaliana

p Value
Total Aphid Proliferation (TAP)
Hours

		12	36	60	84	108
Shoot-(28D)	1 aphid/plant		0.0148	0.0005	0	0
Shoot-(28D)	5 aphid/plant		0.0352	0.0003	0	0
Shoot-(28D)	10 aphid/plant				0	0
Shoot-(35D)	**1 aphid/plant**					**0.0001**
Shoot-(35D)	**5 aphid/plant**				**0.0035**	0
Shoot-(35D)	**10 aphid/plant**				**0.0002**	0
Shoot+(42D)	**1 aphid/plant**			**0.0192**	0.0007	0
Shoot+(42D)	**5 aphid/plant**			**0.0468**	**0.0078**	**0.0014**
Shoot+(42D)	**10 aphid/plant**		**0.0012**	**0**	0	0
Shoot+(49D)	**1 aphid/plant**					
Shoot+(49D)	**5 aphid/plant**				**0.0114**	0
Shoot+(49D)	**10 aphid/plant**				**0.0058**	0

(b)

Arabidopsis thaliana

p Value
Real Time Aphid Proliferation (RTAP)

		12h	36h	60h	84h	108h
Shoot-(28D)	1 aphid/plant		0.0008			
Shoot-(28D)	5 aphids/plant	0.0005				
Shoot-(28D)	10 aphids/plant				0.0001	
Shoot-(35D)	**1 aphid/plant**					
Shoot-(35D)	**5 aphids/plant**				**0.004**	**0.0051**
Shoot-(35D)	**10 aphids/plant**					**0**
Shoot+(42D)	**1 aphid/plant**					
Shoot+(42D)	**5 aphids/plant**	**0.0007**	**0.0092**			
Shoot+(42D)	**10 aphids/plant**		**0.0018**			
Shoot+(49D)	1 aphid/plant					
Shoot+(49D)	5 aphids/plant	**0**				
Shoot+(49D)	10 aphids/plant			**0.0068**	**0**	**0**

Figure 2 *a*, *b*. Aphid clonal propagation in the model plant at different growth stages from three aphid inoculums.

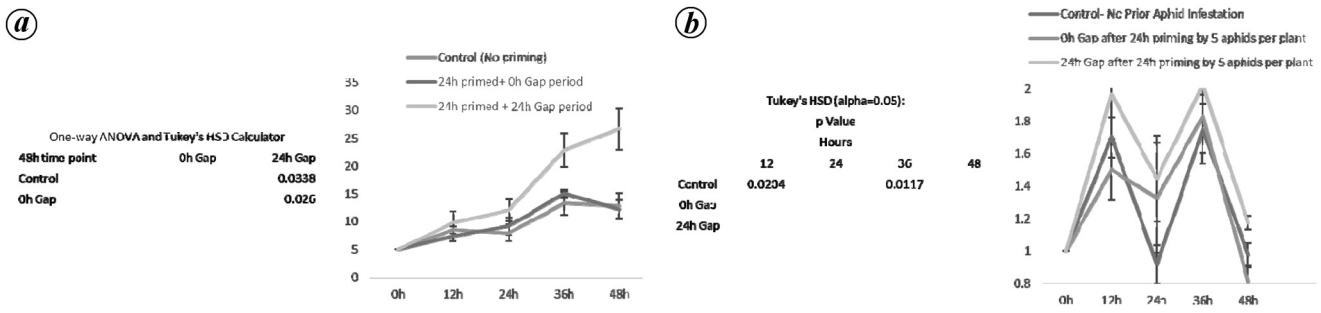

Figure 3 *a*, *b*. Prior aphid herbivory enhanced host susceptibility. Real time aphid proliferation (RTAP) monitoring in aphid herbivory *Arabidopsis* leaf foliage.

enhanced and earliest TAP at 36 h in both doses such as 1 and 5 aphids per plant (Figure 2 *a*). The 35-day-old *Arabidopsis* leaf foliage showed enhanced and earliest TAP at 84 h by both 5 and 10 aphids per plant dose (Figure 2 *a*). The 42-day-old *Arabidopsis* leaf foliage with small emergence of flower stalk showed enhanced and earliest TAP at 36 h at 10 aphids per plant (Figure 2 *a*). The 49-day-old *Arabidopsis* leaf foliage showed enhanced and earliest TAP in 5 and 10 aphids per plant dose. Almost at every development stage, *Arabidopsis* leaf foliage showed enhanced and earliest TAP especially in five aphids release per plant dose (Figure 2 *a*). The RTAP was significant at 12 h by 5 aphids per plant dose in 28-day-old *Arabidopsis* leaf foliage (Figure 2 *b*). Whereas, in 35-day-old plant, the 5 aphids per plant showed the earliest RTAP at 84 h (Figure 2 *b*). In both 42- and 49-days-old *Arabidopsis* leaf foliage, 5 aphids release per plant showed the earliest and significant RTAP at 12 h (Figure 2 *b*). In summary, *Arabidopsis* leaf foliage showed the enhanced and significant TAP and RTAP on different developmental stages of the plant.

The next experiment was formulated to monitor previous aphid herbivory on freshly inoculated aphid proliferation. In this experiment, five aphids were released in each plant and incubated for 24 h. After 24 h, total aphid was counted and found to be non-significant ($P = 0.898$). After removing the aphid, fresh *in planta* bioassay was initiated with five fresh aphids release and abbreviated as 0 h gap. The 24 h gap period was also followed. Interestingly, 24 h gap period showed significant aphid clonal propagation at 48 h as compared to both control and 0 h gap leaf foliage (Figure 3 *a*). The significant RTAP was also comparable in all time points among all primed leaf foliage (0 h and 24 h gap) and control (Figure 3 *b*).

Aphid clonal proliferation was also monitored from different aphid release time points namely at 6 am, 12 noon and 6 pm (Figure 4). From the aphid proliferation record, the aphids released at 6 am showed the earliest and significant TAP at 84 h ($P = 0.0058$) as compared to the initial number of aphid release (Figure 4 *a*). Interestingly, aphids released at 12 noon and 6 pm showed significant aphid proliferation at 108 h (Figure 4 *a*). The significant RTAP was recorded at 12 h in both time points aphid inoculums, i.e. 6 am and 12 noon. Aphid released at 6 am showed significantly earliest time point aphid clonal propagation (TAP) at 84 h which was due to significant RTAP ($P = 0.0025$) at 12 h (Figure 4 *b*).

Presence of flower stalk was considered for evaluating *Arabidopsis* leaf foliage susceptibility (Figure 5 *a*). Aphid inoculum was considered on leaf foliage, not in the flower

stalk portion. Control plant without presence of flower stalk was also considered. The result showed that aphid proliferation was significant at 84 h ($P = 0.0125$) as compared to initial aphid release when flower stalk was present (Figure 5 *a*). Whereas significant aphid proliferation was observed at 108 h in the leaf foliage when flower stalk was absent ($P = 0.0112$) (Figure 5 *a*). Though both *Arabidopsis* with and without flower stalk was not same aged plants, presence of flower stalk enhanced susceptibility on leaf foliage towards aphid clonal proliferation.

The susceptibility of leaf foliage and shoot portion were specifically focused in this experiment. In the previous study, aphids were placed either on leaf foliage or shoot but aphid movement was not restricted within the same plant. Here, 42-day-old *Arabidopsis* having shoot was considered for this experiment. The leaf was removed and the shoot was considered (shoot per leaf) for aphid bioassay. Likewise, shoot was removed and the leaf foliage was considered (leaf per shoot) only for aphid bioassay. Five aphids were released on either shoot or leaf (6 replications). Significant aphid clonal proliferation was recorded at 60 h between leaf per shoot and shoot per leaf ($P = 0.01$; Figure 5 *b*). The significant RTAP was found at 36 h in both and an additional RTAP was also recorded in shoot per leaf at 60 h (Figure 5 *c*). Moreover, significant RTAP difference between leaf and shoot was also observed at 60 h ($P = 0.0211$; Figure 5 *c*). This indicated that aphid could crack the shoot defense much easier than leaf.

Spatial distribution of aphid proliferation in *Arabidopsis* in temporal scale was also monitored (Figure 6). The same aged *Arabidopsis* plant having flower stalk was used for spatial resistance assay. The aphid inoculum was released on the leaf foliage and allowed the aphid to move by host's antagenosis and antibiosis property of the host plant (Figure 6 *a*). The same experiment was also followed by releasing aphid on flower stalk also (Figure 6 *b*). The study showed that aphid colonized at the initial time point at leaf foliage when aphid inoculum was released on leaf portion (Figure 6 *a*). The significant aphid proliferation was realized in leaf foliage at 12 h time point ($P = 0.0069$; Figure 6 *a*). But in temporal time points (36 h and onwards), non-significant aphid colonization was observed between leaf foliage and flower stalk (Figure 6 *a*) when no aphid inoculum was released on flower stalk. When the aphid inoculum was released on flower stalk portion, no aphid colony was established on leaf foliage at any time point (Figure 6 *b*). This is an interesting observation that within the same plant, differential spatial resistance was recorded (Figure 6).

Spatial resistance within whole plant showed that flower stalk was more susceptible than leaf foliage (Figure 6). Experiment was performed to evaluate the spatial resistance within the leaf blade. The aphid colonization was monitored within the leaf blade into mid-rib versus outside mid-rib region at 96 h (Figure 7 *a*). From the result, it was evident that most of the aphid was oriented mid-rib position ($P = 0$) compared to outside mid-rib area (Figure 7 *a*). So, aphid preferred to settle in the mid-rib area. The GUS gene expression driven by ADF and PAD4 promoter was stained in aphid-infested leaf to monitor the expression profile in the mid-rib region where more aphid colonization was established (Figure 7 *b*). The vasculature expression of both gene *pad4* and *adf3* was recorded outside the mid-rib region within the leaf blade (Figure 7 *b*). Interestingly, the expression of *pad4* and *adf3* was not recorded in the mid-rib region due to aphid feeding (Figure 7 *b*). Aphid explored the spatial expression pattern within the leaf blade for colonizing significantly in the mid-rib region (Figure 7 *b*).

The aphid feeding based primed *Arabidopsis* leaf foliage showed the enhanced susceptibility towards aphid clonal proliferation (Figure 3). As the interaction between aphid and host vasculature was established physically between aphid stylet and host SE element cell, aphid-challenged leaf vascular sap was isolated to record the absorbance signature from 190 to 800 nm. From the result, it was evident that the absorbance record in the 210–230 nm range was negative in aphid-challenged leaf foliage vascular sap when aphid un-infested leaf exudate absorbance was

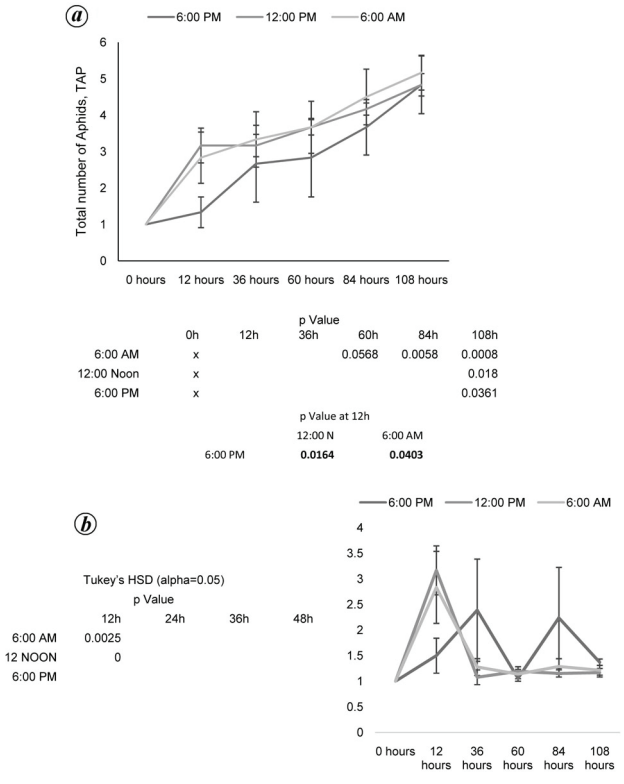

Figure 4. Effect of aphid inoculum timing on temporal clonal proliferation in *Myzus persicae*. One aphid was released in three different time points like 6 am, 12 noon and 6 pm to monitor the aphid clonal propagation in temporal scale. ***a***, Aphid inoculum at different time points showed differential host susceptibility. ***b***, RTAP snapshot of the different temporal time.

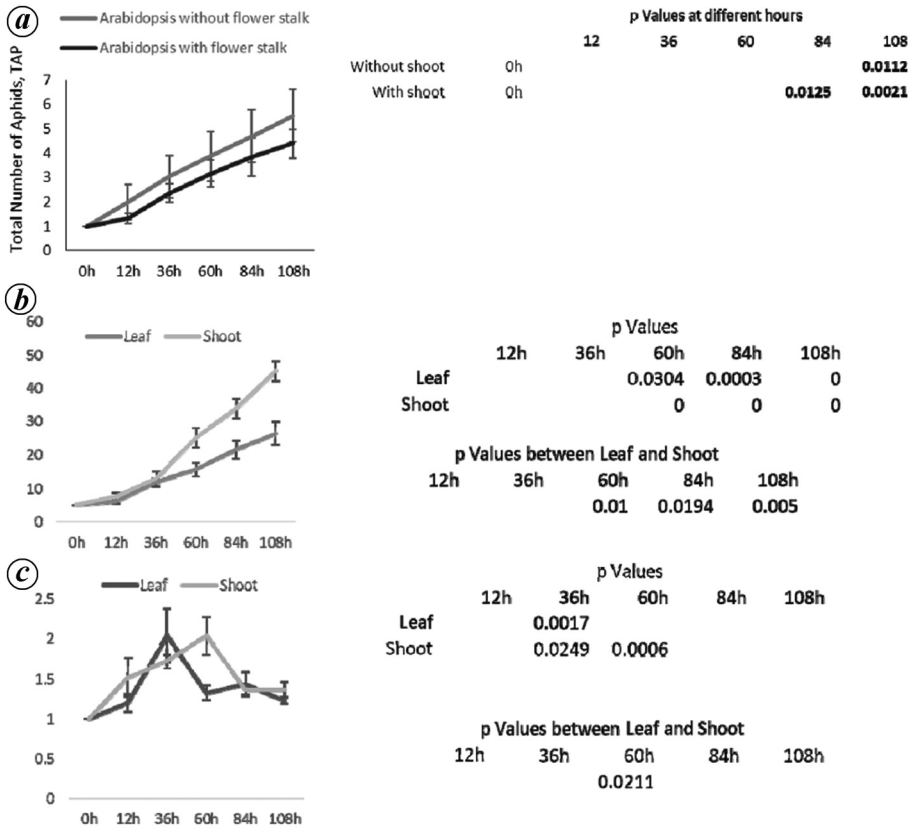

Figure 5 ***a–c*.** The clonal propagation of aphid on the *Arabidopsis* leaf with and without flower stalk.

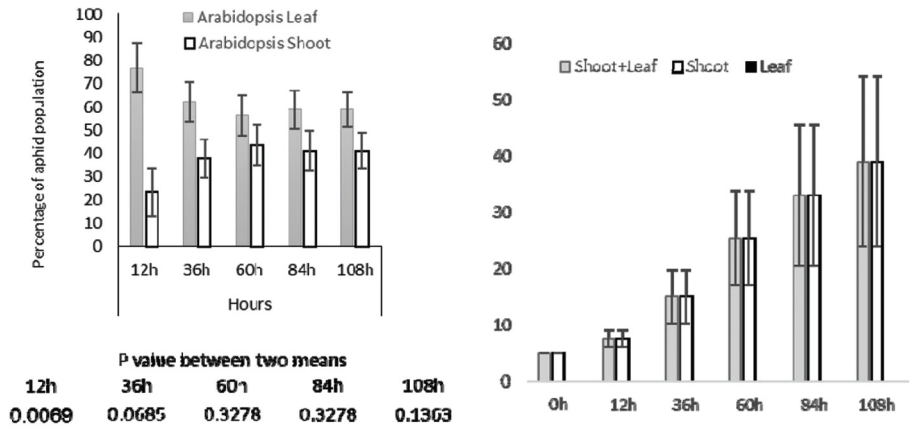

Figure 6 ***a*, *b*.** Spatial Resistance (leaf foliage versus shoot) assay in *Arabidopsis*. ***a***, Spatial distribution of proliferated aphid in *Arabidopsis* when aphid inoculum was released on leaf foliage. ***b***, The relative distribution of proliferated aphids within the host when aphid inoculum was released on flower stalk.

subtracted from aphid-challenged leaf foliage exudate (Figure 8 *a*).

Peptide bonds were absorbed in the 210–230 nm range. The absorbance of –0.438 at 215 nm and –0.307 at 230 nm signified that aphid infestation caused the peptide bond degradation which was reflected in the absorbance signature (Figure 8 *a*). So, it was an indication of degradation of peptide bond or accumulation of amino acids or peptide may be possible reason to explain. The wavelength of maximum absorption for both DNA and RNA is at 260 nm (λ_{max} = 260 nm) and most proteins have a distinct absorption maximum at 280 nm because of the presence of aromatic amino acids (especially tryptophan, tyrosine and phenylalanine). A 260/280 ratio of 1.11 (0.111 at

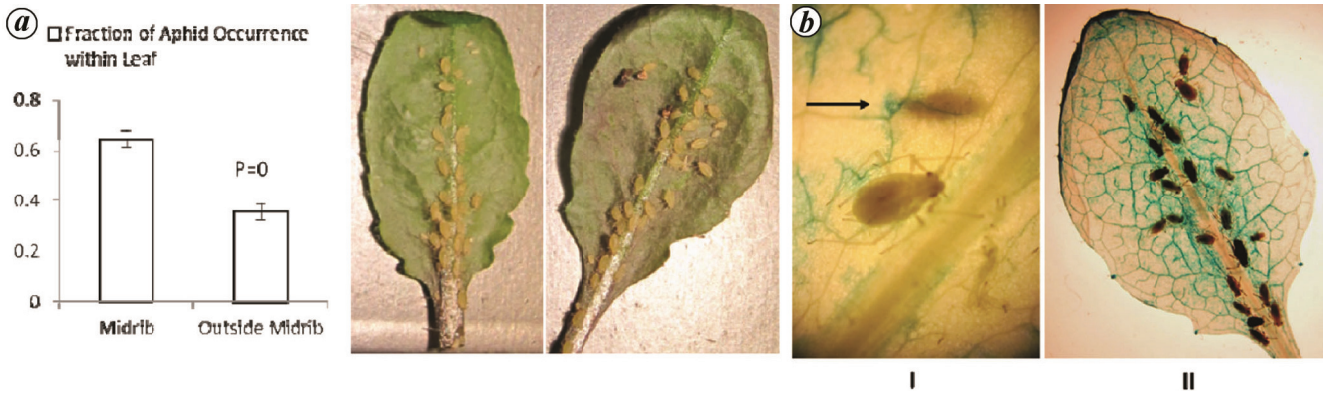

Figure 7 ***a***, ***b***. Monitoring the spatial aphid colony distribution within the same leaf blade. ***a***, Monitoring aphid proliferation spatially within a leaf blade (Aphid's physical orientation to mid-rib and outside mid-rib area for sap sucking). ***b***, Spatial GUS staining assay of aphid-challenged leaf in PAD4-PROMOTER :: GUS and ADF3-PROMOTER :: GUS T2 *Arabidopsis* leaf.

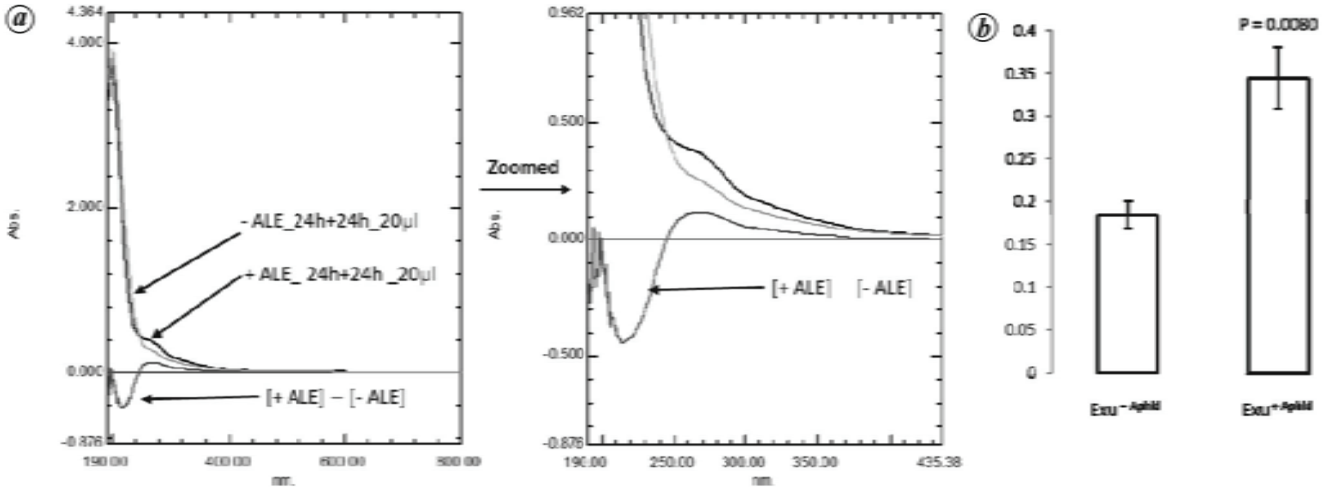

Figure 8 ***a***, ***b***. Absorbance signature (190–800 nm) of aphid herbivory vasculature sap favoured enhanced aphid clonal proliferation in *Arabidopsis* leaf foliage. ***a***, Absorbance signature of the aphid-challenged vascular exudate. ***b***, Bradford assay for the total protein/peptide concentration in the aphid-challenged leaf foliage exudate.

260 nm and 0.1 OD at 280 nm) indicated the presence of more amino acids or degraded peptides in aphid-challenged leaf foliage exudate. Bradford assay was also considered to estimate the total protein/peptide concentration in the aphid-challenged leaf foliage exudates (Figure 6 *b*). Five aphids were released on each *Arabidopsis* plant. After 48 h of incubation, all the aphids were removed. After removal of aphids, leaf exudate was isolated from aphid-challenged foliage. The Bradford assay indicated that aphid-challenged leaf exudate accumulated more basic amino acids (primarily arginine, lysine and histidine), and the number of Coomassie dye (Coomassie G-250) associated bound to positive protein amine groups of each protein molecule/peptide and number of dye associating was approximately proportional to the number of positive charges found on the protein/peptide. In summary, aphid-challenged leaf exudate showed peptide bond degradation of the protein/peptide present in vasculature and the positive charges like basic amino acids in the fragmented protein/peptide were more accessible resulting in significantly higher absorbance in Bradford assay (Figure 8 *b*).

The most important finding from the present study is that a particular aphid inoculum enhanced host susceptibility towards aphid clonal proliferation on the *Arabidopsis* leaf foliage. Here, we have explored three doses of aphid inoculum, i.e. 1, 5 and 10 in each plant. Interestingly, only 5-aphids inoculum caused enhanced host susceptibility (Figure 1). So, particular aphid concentration of aphid number showed this enhanced susceptibility. A similar phenomenon in the bacterial system was evolved and reported as quorum sensing[29]. The term 'quorum sensing' describes cell density of a particular gene expression for specific phenotype regulation, which in turn, coordinates behaviours like biofilm formation, virulence factor expression and motility[30]. Bacteria explored quorum sensing with fulfilling three steps – secretion of a signalling molecule

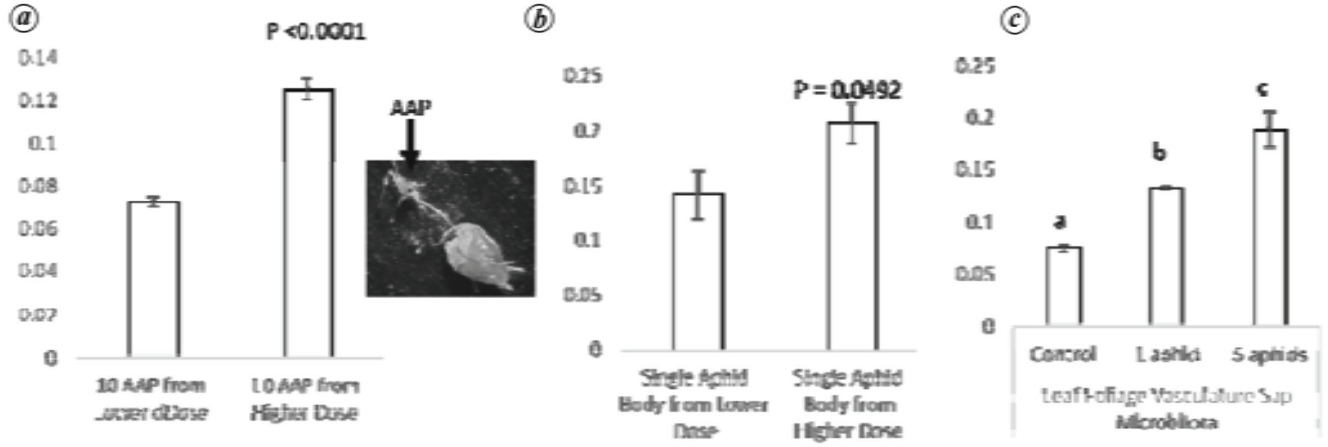

Figure 9 ***a–c.*** Microbiota measure in aphid from lower dose and higher dose aphid-challenged leaf foliage. ***a***, Microbiota from ten numbers of aphid apical part (AAP) containing salivary gland and stylet from lower and higher dose of aphid-herbivore leaf foliage. ***b***, Single aphid microbiota from lower and higher dose of aphid-challenged leaf foliage. ***c***, Aphid-challenged leaf foliage vasculature sap microbiota.

(an autoinducer), detection of the critical concentration of signalling molecules and regulation of gene transcription as a response[31]. The established theme could be potentially applied to plant–aphid interaction biology. The quorum number of aphids (here, 5 aphids per plant inoculum) could enhance host susceptibility. So, a quorum number of aphids present on *Arabidopsis* leaf foliage might communicate to other aphids positioned on the *Arabidopsis* leaf foliage as the aphid inoculum followed random position on the leaf foliage. The concentration of signalling molecules (autoinducers in bacterial system) modulated in response to changes in aphid-population density needs to be uncovered. The interaction among aphids for quorum sensing on *Arabidopsis* leaf foliage which showed enhanced host susceptibility towards aphid clonal propagation needs to be studied at the molecular level. This mechanism could be uncovered at the molecular level by identifying the molecules either secreted by aphid feeding or interaction product or plant specific molecule specifically in the model plant, *A. thaliana*. The quorum number of aphid population was varied on different developmental stages of *A. thaliana* (Figure 2). The aphid benefitted from the primed *Arabidopsis* leaf foliage which was primed by the quorum number of aphids feeding based incubation (Figure 3). Aphid also explored the weak space of the leaf blade where no aphid resistance gene was expressed (Figure 7 *b*). Aphid feeding based priming degraded the peptide bond and enhanced RNA molecule also favoured the fresh aphid to proliferate rapidly (Figures 3 and 8). The secondary (facultative) endo-symbiont such as *Serratia symbiotica* was also housed in the aphid body. It was reported that many *Serratia* species secrete extracellular enzymes[32,33]. *S. symbiotica* may produce proteases which may facilitate the digestion of plant proteins, and thereby suppressing plant defense[32,33]. We realized that the peptide bond degradation in aphid-challenged leaf foliage (Figure 8) could facilitate the fresh aphid to proliferate at enhanced rate as compared to control plant (Figure 3). Another line of experiment, we linked that microbiota from 10 numbers of AAP from higher dose (@ 5 aphid per plant) of aphid-challenged leaf foliage showed significantly higher microbiota as compared to lower aphid inoculum (@ 1 aphid per plant) (Figure 9 *a*). Even, single aphid from higher dose (@ 5 aphid per plant) of aphid-challenged leaf foliage vasculature showed an enhanced concentration of microbiota as compared to lower dose (@ 1 aphid per plant) of aphid inoculum at 36 h (Figure 9 *b*). Aphid-challenged leaf foliage vasculature sap microbiota was also significantly enhanced in a higher dose (@ 5 aphid per plant) as compared to the lower dose (@ 1 aphid per plant) (Figure 9 *c*). This experiment indicated new perspective[34] of aphid inoculated microbiota based enhancing host susceptibility towards aphid clonal proliferation from a particular aphid inoculum.

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