Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(6): 1270-1276

Received: 02-09-2018
Accepted: 05-10-2018

Kamrul Hassan
(1) Department of Entomology, Faculty of Agriculture, Sylhet Agriculture University, Sylhet-3100, Bangladesh
(2) PhD Candidate, Hawkesbury Institute for the Environment, Western Sydney University, Australia

Nasrin Akter
Department of Entomology, Faculty of Agriculture, Sylhet Agriculture University, Sylhet-3100, Bangladesh

Md. Fuad Mondal
Department of Entomology, Faculty of Agriculture, Sylhet Agriculture University, Sylhet-3100, Bangladesh

Mahfuza Pervin
Faculty of Science, University of Copenhagen, Denmark

Correspondence
Kamrul Hassan
(1) Department of Entomology, Faculty of Agriculture, Sylhet Agriculture University, Sylhet-3100, Bangladesh
(2) PhD Candidate, Hawkesbury Institute for the Environment, Western Sydney University, Australia

Foliar spray of benzoic acids on pollinators behavior and plant fitness benefits

Kamrul Hassan, Nasrin Akter, Md. Fuad Mondal and Mahfuza Pervin

Abstract
Induction by herbivores either positively or negatively effect on pollinators behavior. However, there are likely to be little studied whether foliar spray of Benzoic acids on plant can be correlated with pollinator behavior. Our studies focus on the application of benzoic acid at 0mM, 0.005mM, 0.05mM, 0.5mM and 0.1mM doses respectively on *Brassica nigra* at flowering stage. Common garden experiment was performed with five treatments while each replicated six times. Overall, honey bee's attraction was significantly higher at 0.1mM than control plots albeit honey bees visiting flowers were similar to control plants. We assumed there were no changes which would affect the attraction of pollinators or changes could be perceived but there was no reason to change the behavior. However, at 0.05mM, syrphid flies visited more flowers than control plots and variation was statistically significant. Pod and seed weight (gm) was increased at 0.01mM than control. Our result indicates that at high doses pollinator visited more flower than control, therefore is likely to increase seed weight (gm).

Keywords: HIPVs, benzoic acid, pollinators attraction and visitation, plant fitness

1. Introduction

Plant chemical defense to herbivory by producing wide range of toxins and volatiles cues [1, 2]. These volatile cues linked the interactions of plant and carnivore with herbivore called herbivore-induced plant defenses (HIPDs) [3]. However, plant gets advantage while herbivory attenuate pollinators attraction and also likely to enhance predator and parasitoid recruitment for other herbivory [4]. In essence, plant defense not only aid them to get rid from herbivore attack but also accomplishing interaction with pollinators. As because herbivory changes the physiology, morphology and reproductive traits of plants, most distinctively floral display and nectar quality of flowers [5].

On the eve of plant responses to herbivores can eventually affected flower traits and consequently change pollinator behavior [6, 7] As animal pollination is an active process for the plant: animals need to be lured by plants do that they can transport pollen from one flower to the next. The multiple flower traits evolved in plants and exploited by pollinators can be divided in long and short-range cues. Long range cues are flower morphology (size and symmetry), number of flower, color and rate of scent emission [8]. Short range cues are quality and quantity of nectar occurring defensive secondary metabolites [9, 10]. Pollinators use both long and short range cues, and often multiple at the same time to choose which flowers to feed from [11].

The secondary metabolites produced in nectar mostly for plant defense can be grouped into alkaloids, phenolic and other non-protein amino acids [12]. Among secondary defensive metabolites, alkaloids are the most diverse group and approximately 12,000 structured already possible to explained [13]. In alkaloids, benzoic acid $C_7H_6O_2$ is a colorless crystal solid and a simple aromatic carboxylic acid [14]. Benzoic acid may occur naturally in many plants and act as an mediator of biosynthesis of many secondary metabolite [15]. Some previous studies show that caffeine impede to honey bees at high doses [16]. It is assume that caffeine pharmacologically change the behavior of pollinator by augmenting their memory of reward [17]. For instances, honeybees rewarded with caffeine found in the nectar of *Coffea* and *Citrus* species, possessed three times higher attraction of floral reward than only reward with sucrose [17]. Although low doses of caffeine had less impact on short term memory whereas very effective in long term memory. It indicated that low doses of caffeine did not affect the bee's intention for the selection for nectar [17]. However, to be knowledge, there were few studies done so far how benzoic acid and other purine alkaloids affects the pollinator attraction, pollinator community composition, and efficiency of pollen transfer.

Our goal to investigate to various benzoic acid concentration level occurred in nectar, to test if and how pollinator responses to benzoic acid, and whether responses of pollinator vary with benzoic acid concentrations as well as what changed occurred in nectar reward. So, in our study benzoic acid was applied as foliar spray to assess the performance of pollinators behavior. The study was accomplished to answer the research questions: Does benzoic acid affect bees' foraging behavior by altering the food source; nectar or pollen reward? and Does the foliar spray of benzoic acid increase the plant reproductive success and what level of concentration, it will reach maximum success? Our hypothesis was pollinator behavior may alter among the benzoic concentration level. As different concentration level of benzoic acid would attract pollinator differently due to perception of cues from low to high dose. Some studies have shown that upon herbivore attack the level of secondary metabolites increases and it causes more visitation by honey bees than control plant [11]. We may predict artificial application of benzoic acid may be increased the pollinators performance as it was increased in caffeine [16].

2. Materials and Methods

The experiment was conducted to field site of Sylhet Agricultural University, Bangladesh during winter season, 2017. The experimental soil was slightly acidic to strongly acidic in nature [18]. The land was prepared on 10 November 2017 by breaking all the clods as well as level the land as much as possible. Weeds, stubbles and other residues were removed from the field. Field layout was done by pseudo Latin Square Design (LSD). Individual plots or beds were prepared with spade. The length and width of the bed was 1.25m & 1.15m respectively and the gap between two beds were 30cm and the excavated soil from field was used for raising the plots to about 5 cm high from the soil surface. Seeds were sown on 13 November 2017. After sowing, the seeds were covered with the soil, and bamboo leaf and slightly pressed by hand to protect from direct sunlight.

2.1 Plant

The Black Mustard, (*Brassica nigra* L., Brassicaceae) was used as tested plant. It is an annual plant species and produces hermaphroditic flowers [19]. It is largely depending on insects for pollination but can also make selfing when herbivore attack the plant. The pollinator community consists of honey bees, bumblebees, syrphid flies, many species of solitary bees and butterflies. Seeds of *B. nigra* were obtained from the local germplasm of Sylhet.

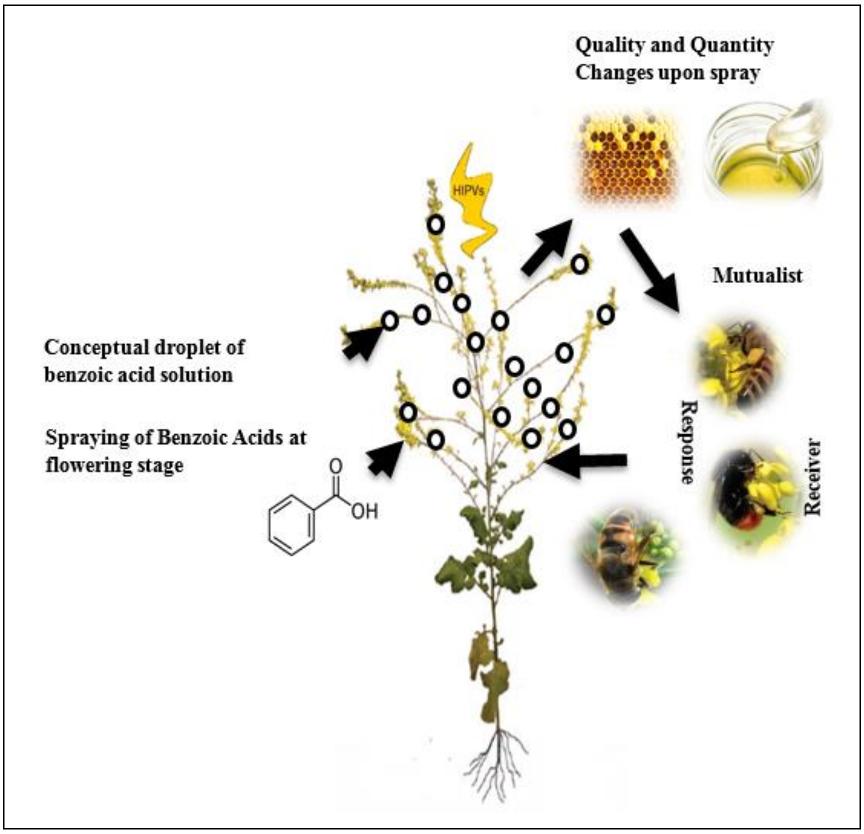

Fig 1: Schematic representation of the study system.

2.2 Common garden experiment

Common garden experiment layout consists of 30 plots of *B. nigra* plants. Each of the plot was composed by 10 plants. The distance between the central plant and the 4 side plants was of 20 cm. The distance between plots of 5 plants was 1.16 m. The experiments were designed by using a pseudo Latin square design, thus plots of plants subjected to the same treatment were never planted in the same row or Colum. The Control=0mM; here, A=control, Treatment (B) = 0.1mM, Treatment (C) = 0.5mM, Treatment (D) = 0.05mM and Treatment (E) = 0.005mM.

2.3 Treatment and preparation of benzoic acid solution

Benzoic acid was applied in the field at four different concentration including a control treatment. The concentrations were 0.005mM, 0.05mM, 0.1mM, and 0.5mM, respectively. The concentration of control treatment was 0.0mM, only water was sprayed in the plot. Each of the concentration was considered as treatment. The whole experiment was consisting of 5 treatments. Each of the treatment was replicated 6 times. The following equation was followed to make 0.005 mM Benzoic Acid solution. The rest of the solutions were also prepared by underneath calculation.

Mass [g] = molecular weight [g/mol] * concentrations [mol/L] * volume [L]
= 122.12 g/mol * 0.000005 mol/L * 1000 ml
= 0.61 g
= 0.30 g / 500 ml

2.4 Preparation of spray solution

The calculated 0.3 g benzoic acid was added to 500 ml distilled water in a 500ml beaker. The beaker was placed in Magnetic Stirrer to mix the benzoic acid properly. It was taking around 2 hours to make the final solution of benzoic acid. For the foliar spray, a hand sprayer was used to proper discharge of the solution. For proper application, each of the spray considered 2ml of benzoic acid solution. Each of the plot in the field consists of 10 plants. Each plant received 5 spray which was equivalent to 10 ml solution. So, each plot received 100 ml solution of benzoic acid see details in figure 1.

2.5 Selection of plant stage for foliar spray

B. nigra pass through three distinct plant ontogenetic stage; vegetative, bud and flowering stage. For foliar spray flowering stage was considered. We recorded when the first flower of each plant open, and when 8 out of 10 plants had flowers, the plot was considered to be flowering, then foliar spray was done.

2.6 Data recording

Data was recorded on number of pollinator visited, number of flower visits, visitation time (s) mainly on honeybees (*Apis indica*) bumble bees (*Bombus sp*.), syrphid flies (*Eristalis sp*.), and solitary bees, butterflies and another possible pollinator of *Brassica nigra*. We also collected the data of pod weight (gm) and seed weight (gm) for each plot.

2.7 Observation of pollinators

Pollinator observations took place at one-time points; one day after foliar spray. Each plot was monitored for 10 minutes. When a pollinator entered the plot and landed on a flower, the identity of the pollinator, number of flowers visited and visitation time (s) was recorded. If during the observation of an individual pollinator, other pollinators entered the plot, their visitation and identity was recorded. If the same individual pollinator returned to the plot under observation after having visited a different plot, we recorded that visit as a new visit. Pollinator identity was recorded as: honeybees (*Apis sp.*), syrphid flies (mainly *Eristalis sp.* but including other Syrphidae), and solitary bees (any Apidae except *Bombus* sp.). Observations were carried out between 10 am and 1 pm when weather conditions were suitable (Temperature; 15-30ºC and wind speed ≤ 6 m sec^{-1}).

2.8 Data analysis

Analysis of variance (ANOVA) was done to see the significant differences between the treatment for pollinators community analysis, total pollinator attraction and flower visits. To see the difference between the treatments for individual pollinator attraction and flower visit ANOVA was also done. One-way ANOVA was also done for pod weight and seed weight (gm) respectively.
The analysis was done in R version 3.5.0 (2018-04-23).

3. Results

3.1 Total pollinator communities and their flower visits

We recorded total 823 pollinators and 2294 flower visited by those pollinators (belongs to the group of honey bees, solitary bees, bumble bees, syrphid flies, butterflies and other flies) in entire experiment. Our results showed that, pollinator attraction and number of flower visited was not significant than control plots among doses (total pollinator attraction; One-way ANOVA P = 0.541, df=4; and total flower visit; One-way ANOVA P = 0.402, df=4). Albeit at 0.1 and 0.5 mM doses the pollinator attraction and flower visitation was higher than control plots (figure 2).

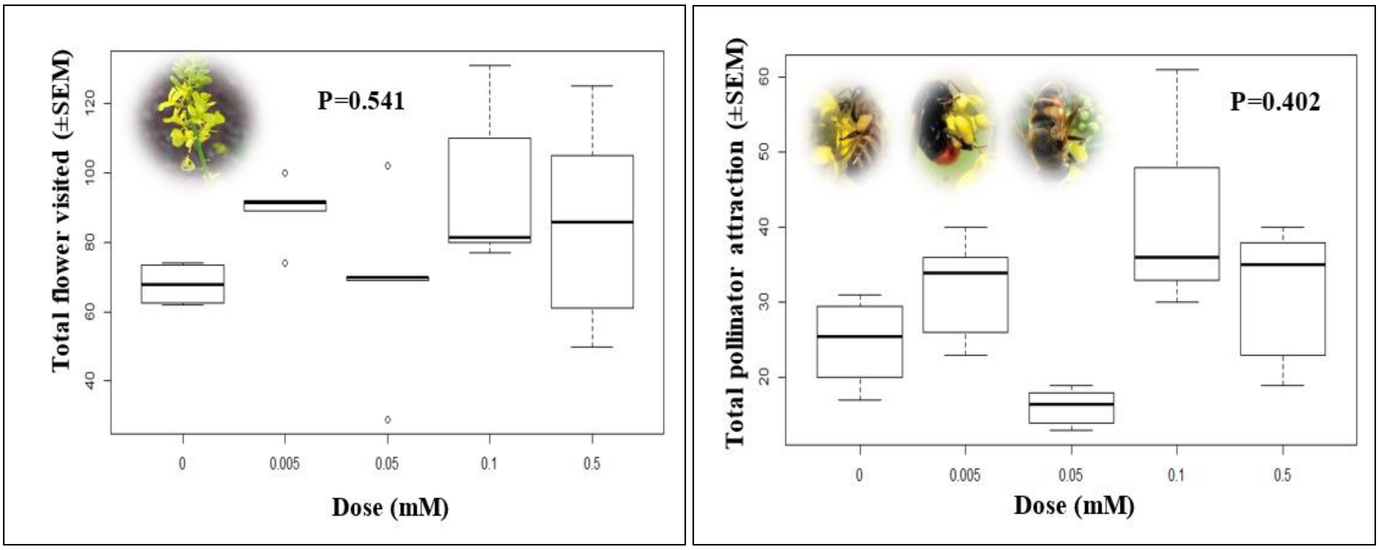

Fig 2: Total number of pollinators (mean ± SEM) and flower visited by pollinator communities (mean ± SEM) to plots of *B. nigra* plant in response to foliar spray of Benzoic acid. Data was analyzed with One-way ANOVA at 0.05 and 0.01 significance level. Error bar represents ± SEM.

3.2 Attraction of honey bees and flower visited

We observed 659 honey bees both treated and untreated plots. We tested whether there was an effect of foliar spray of benzoic acids at various doses on honey bees attraction. The highest number of honey bees attraction was recorded at 0.1mM doses however, it was not statistically significant than control plots (honey bees attraction; One-way ANOVA P = 0.541, df=4). Surprisingly, at 0.05mM dose attraction of honey bees was lower than control plots. Although honey bees attraction was significant between 0.05 and 0.005 (One-way ANOVA P <0.05, df=1) and 0.05 and 0.1 mM doses (One-way ANOVA P <0.05, df=1; figure 3). In total there were 2156 flower visited by honey bees both treated and control plots. Overall, the highest number of flower visited by honey bees at 0.005mM and 0.1mM doses respectively but there was no significant variation than control plots (One-way ANOVA P = 0.541, df=1).

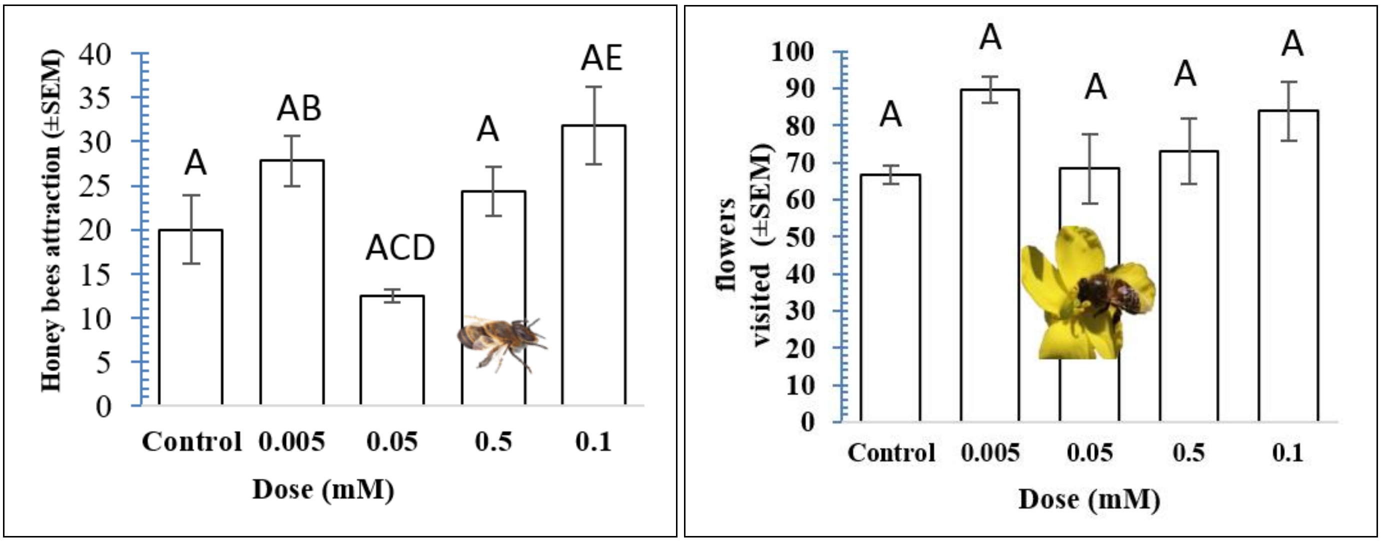

Fig 3. Attraction and flower visited by honeybees (mean ± SEM) at various concentration level. Data was analyzed with ANOVA at 0.05 and 0.01 significance level. Error bar represents ± SEM. Different upper case later indicated significant differences between the treatments and same letter indicated there were no significant difference between the treatments.

3.3 Syrphid flies attraction and flower visited

Only 37 syrphid flies and 92 flower visited by them both treated and control plots. Overall, highest number of syrphid flies attraction was recorded at 0.1mM doses of benzoic acids but not statistically significant than control plots (One-way ANOVA P = 0.252, df=4). However, at 0.05 and 0.005 dose the attraction of syrphid flies were almost likely to be control plots. On the contrary, highest number of flower visits were recorded at 0.5mM of benzoic acids which was statistically significant than untreated plots (One-way ANOVA; Overall, P = 0.0466, df=4; and between treatments; P<0.001, df;1). However, there were almost similar flower visits observed rest of the doses (figure 4).

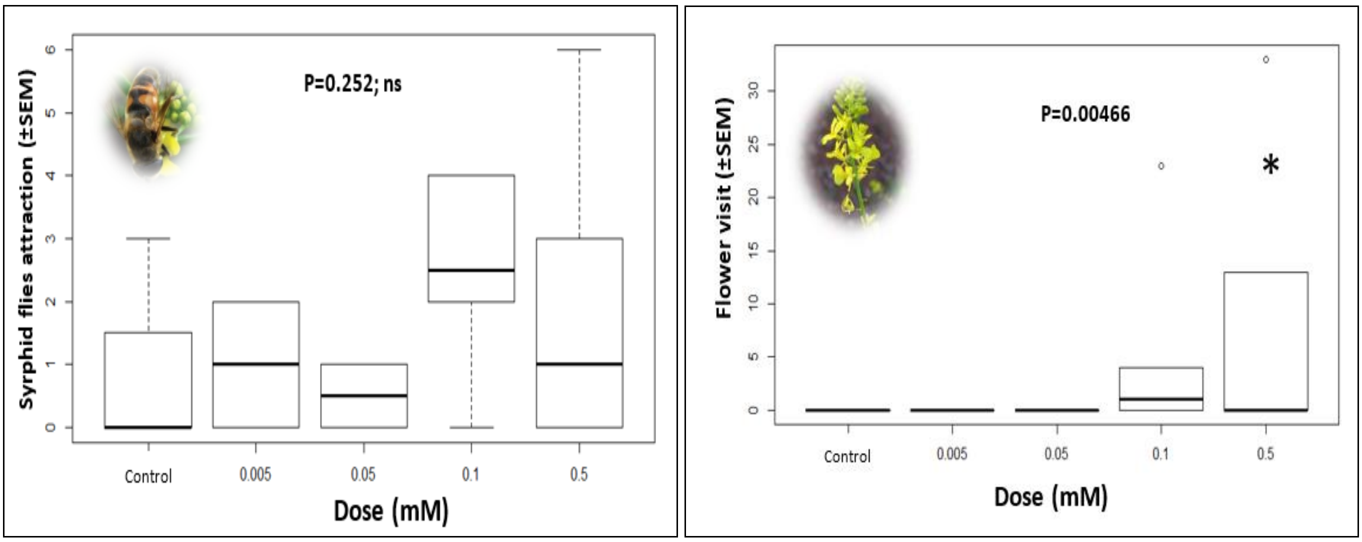

Fig 4: Syrphid flies attraction and flowers visited (mean ± SEM) to plots of *B. nigra* plant in response to various concentration level. Data was analyzed with ANOVA at 0.05 and 0.01 significance level. Error bar represents ± SEM. Asterisk indicate the significant difference between the treatments and ns indicated non-significant differences between the treatments.

3.4 Other pollinator attraction and flower visits

Overall 27 solitary bees and 42 flower visits were recorded in the experimental period. Data were not sufficient for statistical analysis. We assumed that solitary bees were very rare in Bangladesh and few species are in danger. Our research indicated that population of solitary bees might be decreased and thenceforth less visits of solitary bees in the plots. Bumble bees attraction was least in experimental field. Several types of butterflies may also assist in pollination although their role is very minor in contrast to other pollinator. We recorded very less number of butterflies that visited different plots of *B. nigra*.

3.5 Pod weight and Seed weight (g)

Overall, our results showed that due to variation in pollinator responses, plants responded differently in their reproductive success. The highest number of pod weight (gm) was found in 0.1 mM doses irrespectively to all treatments. However, the pod weight was not significant at 0.1 doses than control plots (One-way ANOVA P = 0.468, df=4). Similarly, seed weight (gm) was highest at 0.1mM doses than all other treatments however increased seed weight was not significant than control plots. (One-way ANOVA P = 0.831, df=4; figure 5)

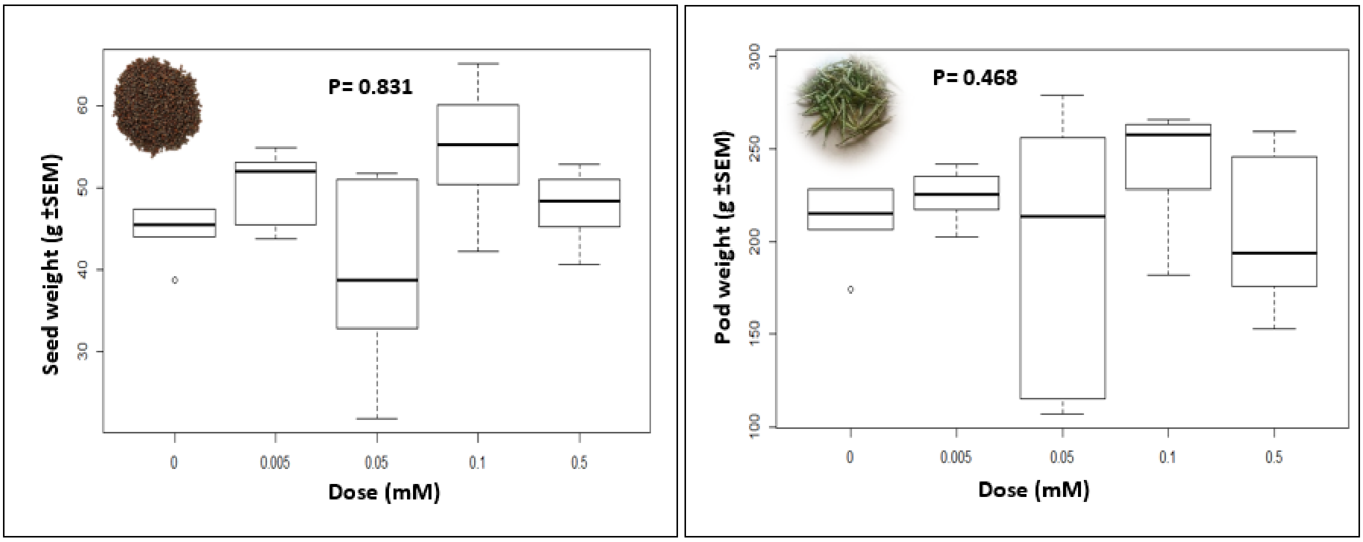

Fig 5: Pod weight (g) and seed weight (g) (mean ± SEM) of three plants of each plots *B. nigra* in response to various concentration level. Data was analyzed with ANOVA at 0.05 and 0.01 significance level. Error bar represents ± SEM.

4. Discussion

Our main objective of this study to evaluate foliar spray of benzoic acids on pollinator foraging behavior in *B. nigra*. In this study, we measured how different pollinator behave at various concentration level of benzoic acids. Furthermore, we also linked how attraction and visitation of pollinator communities and individual pollinator enhance plant fitness benefits via doses of benzoic acids. Overall, our results suggest that at high doses; 0.01mM and 0.5mM, perception of pollinator communities as well as sole pollinators were varied albeit most the results were very close to be significant (Table 1). Moreover, such perception of pollinators has increased the yield; pod weight (g) and seed weight (g) at higher doses.

Table 1: Overall effects of pollinator foraging behavior at different benzoic acids doses. P-value indicated the significant effects of the Benzoic acid doses.

Pollinator behavior	Overall Effects	Benzoic acid Treatments	Significant Effects (*p-value*) **
Total pollinator attraction	Neutral	Increased at 0.1mM	P=0.402
Total pollinator flower visit	Neutral	Increased at 0.1mM	P=0.541
Total honey bee's attraction	Positive	At 0.1mM	P=0.001*
Total honey bees flower visit	Neutral	Variable in doses	P=0.252
Total Syrphid flies attraction	Neutral	Variable in doses	P=0.2.52
Total Syrphid flies flower visit	Positive	At 0.1mM	P=0.004*
Overall pod weight (gm)	Neutral	Increased at 0.1mM doses	P=0.468
Overall seed weight (gm)	Neutral	Increased at 0.1mM doses	P=0.831

**The effects were positive only at high doses 0.1mM but at low doses there were much not variation observed than control and the effect was not visible. So, pollinator might be perceived the message at low doses but there was no reason to response.

4.1 Pollinator communities and their flower visits

Our results revealed that, pollinator communities were responded differently with doses. On the other hand, the overall visitation remains variable according to the foliar spray of benzoic acids. We speculated that each of pollinators perceived the message from benzoic acids doses differently and consequently attraction and visitation was anomalous [9].We found highest visit for total pollinator communities in high doses of Benzoic acids. Our result was supported by [17] while the found honey bees perceived the message from high caffeine doses in nectar that low doses.

We assumed that variation in doses changes the quality and quantity of nectar as like as other studied also revealed [20, 21]. According to [22] aphid infested plots have highest visit of pollinator due to increase sweetness of nectar. By contrast, we did not infest our plots by herbivore, but highest doses may increase the sweetness of nectar and henceforth pollinators visits were increased with the increase of Benzoic acid doses [23, 24]. However, excluding honeybees and syrphid flies we had not enough visits for solitary bees, bumble bees and other pollinators. We assumed that may be at dose variation they received the message as cues but there was no reason to them to responses while honey bees and solitary bee's responses well enough [25]. Moreover, they will not have interested with benzoic acids but would be respond with other secondary metabolites [26].

4.2 Correlation between flower visitation versus pod and seed weight (gm)

Success of pollination by a pollinator depends on how efficiently pollen is transferred to stigma. Therefore, increase visitation of pollinators theoretically means efficiency of pollination but in practical however it is variable. Our study aims to enhance the visitation by adding supplementary metabolites; benzoic acid at flowering stage of *B. nigra* through foliar spray. Although pollinators are responded differently which usually they do, with the doses but what extent it is influenced pod and seed weight (gm) would be the matter. Pearson correlation between flower visits and pod and seed weight (gm) indicated that seeds weight have positive correlation with flower visits (t = 2.7266, df = 26, p-value = 0.005652 at 99% Confidence interval) albeit pod weight not displayed significant correlation (t = 1.4223, df = 26, p-value = 0.08342 at 99% *CI*; figure-6). However, with this study, we can at least assume that spraying secondary metabolites would be benefitted for plant fitness as seed weight is positively correlated with pollinator flower visitation.

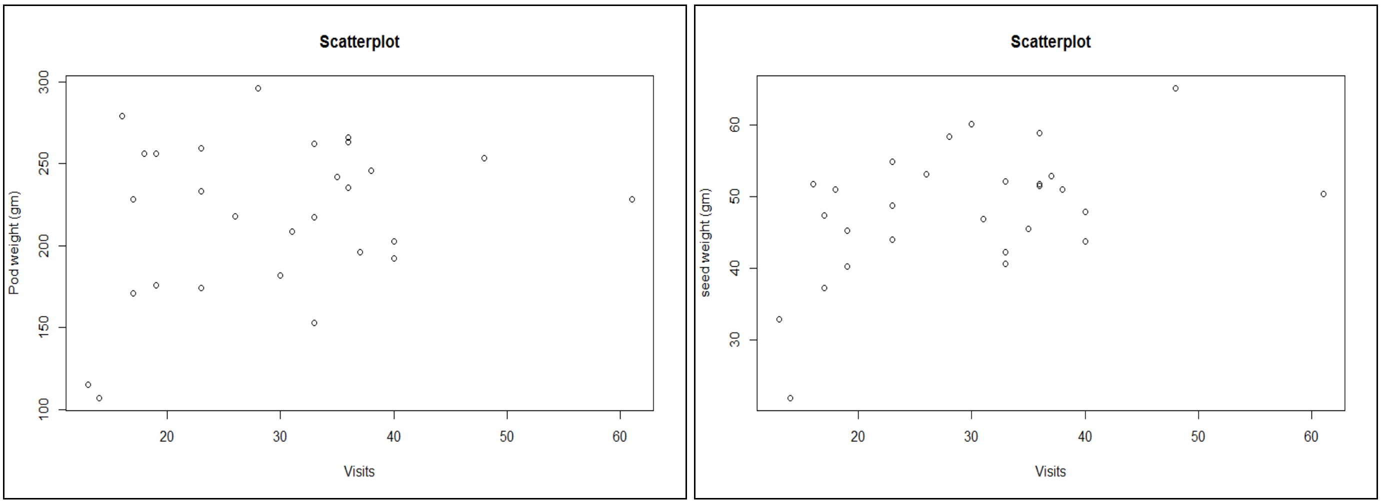

Fig 6: Pearson correlation between flower visitation and pod and seed weight (gm).

4.3 Plant fitness benefits

Our results indicated that plants had benefited upon foliar spray as we found at 0.1mM dose there were highest pod weight and seed weight (gm). We assumed that at higher doses pollinator perceived the changes in nectar and they tend to visit more on that plant. As pollination process is the key to success pod formation and number of seed in any plants and it is completely depending on pollinator itself so we had the highest at 0.1mM that eventually supported by [7, 26]. Albeit, at 0.5mM doses we had less pod weight and seed weight than 0.1mM doses. We speculate that at 0.05mM doses might be cross the threshold level and at such doses, nectar could be the toxic for pollinators. Henceforth, we had lowest fitness benefits at 0.5mM dose than 0.1mM dose.

5. Conclusion and future direction

This is the first study of foliar spray of secondary metabolites to evaluate pollinators attraction and visitation in our knowledge. Therefore, application of multiple secondary metabolites with various doses at greenhouse and field conditions will need to design for future research. We should focus on changed in flower traits (morphology, nectar and pollen composition) and pollination efficiency of different pollinators group in responses to foliar spray of different secondary metabolites. It can help to select best one to spray in the field to augment the pollination services by bees and flies for sustainable agricultural production.

6. Author Contributions

KH and NA design and conduct the experiments. KH and MP conduct data analysis and wrote the manuscript. MP and FM review the article. There is no conflict of interest.

7. Acknowledgement

The authors are grateful to the University Grants Commission of Bangladesh (UGC) and Sylhet Agricultural University Research System (SAURES) for providing financial support.

8. References

1. Gols R *et al.*, Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild Brassica populations. Journal of chemical ecology, 2008; 34(2):132-143.
2. Hopkins RJ, van Dam NM, van Loon JJ. Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annual review of entomology, 2009, 54.
3. Dicke M, Baldwin IT. The evolutionary context for herbivore-induced plant volatiles: beyond the ‘cry for help’. Trends in plant science. 2010; 15(3):167-175.
4. Barber NA *et al.*, Herbivory reduces plant interactions with above-and belowground antagonists and mutualists. Ecology. 2012; 93(7):1560-1570.
5. Barber NA, Soper Gorden NL. How do belowground organisms influence plant–pollinator interactions? Journal of Plant Ecology. 2014; 8(1):1-11.
6. Kessler A, Halitschke R. Specificity and complexity: the impact of herbivore-induced plant responses on arthropod community structure. Current opinion in plant biology. 2007; 10(4):409-414.
7. Lucas-Barbosa D, van Loon JJ, Dicke M. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry. 2011; 72(13):1647-1654.
8. Hoffmeister M, Wittköpper N, Junker RR. Herbivore-induced changes in flower scent and

morphology affect the structure of flower–visitor networks but not plant reproduction. Oikos. 2016; 125(9):1241-1249.
9. Bischoff M *et al*., Context-dependent reproductive isolation mediated by floral scent and color. Evolution, 2015; 69(1):1-13.
10. Tiedeken EJ *et al*., Bumblebees are not deterred by ecologically relevant concentrations of nectar toxins. Journal of Experimental Biology. 2014. jeb. 097543.
11. Lucas-Barbosa D. Integrating studies on plant–pollinator and plant–herbivore interactions. Trends in plant science. 2016; 21(2):125-133.
12. Adler LS. The ecological significance of toxic nectar. Oikos, 2000; 91(3):409-420.
13. Detzel A, Wink M. Attraction, deterrence or intoxication of bees *(Apis mellifera)* by plant allelochemicals. Chemoecology, 1993; 4(1):8-18.
14. Natella F *et al*., Benzoic and cinnamic acid derivatives as antioxidants: Structure-activity relation. Journal of agricultural and food chemistry. 1999; 47(4):1453-1459.
15. Khadem S, Marles RJ. Monocyclic phenolic acids; hydroxy-and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. Molecules. 2010; 15(11):7985-8005.
16. Mustard JA *et al*., Consumption of an acute dose of caffeine reduces acquisition but not memory in the honey bee. Behavioural brain research. 2012; 232(1):217-224.
17. Wright G *et al*., Caffeine in floral nectar enhances a pollinator's memory of reward. Science. 2013; 339(6124):1202-1204.
18. Makin AA *et al*., Ecological diversity and abundance of earthworms in sylhet metropolitan area of Bangladesh. Advances in Zoology and Botany. 2014; 2(4):63-68.
19. Conner JK, Neumeier R. Effects of black mustard population size on the taxonomic composition of pollinators. Oecologia. 1995; 104(2):218-224.
20. Adler L. Selection by pollinators and herbivores on attraction and defense. Specialization, speciation and radiation: The evolutionary biology of herbivorous insects. 2007, 162-173.
21. Adler LS, Karban R, Strauss SY. Direct and indirect effects of alkaloids on plant fitness via herbivory and pollination. Ecology. 2001; 82(7):2032-2044.
22. Hassan K. Effect of different herbivore induction in plants of different developmental stages, and consequences for flower-associated organisms, 2016.
23. Aizen MA, Harder LD. Expanding the limits of the pollen-limitation concept: effects of pollen quantity and quality. Ecology. 2007; 88(2):271-281.
24. Lerch-Henning S, Nicolson SW. Bird pollinators differ in their tolerance of a nectar alkaloid. Journal of avian biology. 2013; 44(4):408-416.
25. Kessler D, Baldwin IT. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of Nicotiana attenuata. The Plant Journal. 2007; 49(5):840-854.
26. Lucas-Barbosa D *et al*., Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production. Functional Ecology. 2013; 27(1):245-254.