Journal of Agriculture and Natural Resources (2021) 4(2): 98-110
ISSN: 2661-6270 (Print), ISSN: 2661-6289 (Online)
DOI: https://doi.org/10.3126/janr.v4i2.33677

Research Article

Effects of different mulches and net house on crucifer aphid (*Brevicoryne brassicae* L.) population, growth and yield of broadleaf mustard (*Brassica juncea*)

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Received: June 01, 2020; Revised: October 30, 2020;
Accepted: December 15, 2020; Available online: January 01, 2021

ABSTRACT

Crucifer aphid, *Brevicoryne brassicae*, is a key pest of broadleaf mustard and other crucifers. An alternative integrated management approaches are recommended to keep the pest below economic threshold level. A field experiment was carried out to evaluate the effect of mulching and net house on aphid population, growth and yield of broadleaf mustard. Experiment was carried out in randomized complete block design with four replications from September to December 2016 at Rampur, Chitwan, Nepal. Five treatments used in experiment was untreated control, black plastic mulch only, reflective plastic mulch only, black plastic mulch plus imidacloprid 70 WSG @ 0.13g/L, and net house plus black plastic mulch. The results showed that the lowest population of crucifer aphid was recorded inside the net house with black plastic mulch and black plastic mulch with imidaclorpid 70 WSG @ 0.13g/L spray. Reflective plastic mulch was superior as compared to black plastic mulch and control to reduce the aphid population. Similarly, the highest yield (26.86t/ha) was obtained inside the net house with black plastic mulch followed by black plastic mulch with imidacloprid spray (25.99 t/ha). But the benefit-cost ratio was the highest (4.09) in black plastic mulch with imidacloprid spray followed by reflective plastic mulch (3.42), black plastic mulch (3.32), and net house with black plastic mulch (3.10). Benefit-cost ratio was lower in net house with black plastic mulch but products are safe from toxins and potentially profitable in long run. Considering its ecological cost, the use of pest exclusion net is recommended as a viable option for controlling insect pests of broadleaf mustard.

Keywords: Broadleaf mustard, net house, crucifer aphid, reflective plastic mulch, black plastic mulch

Correct citation: Regmi, R., Poudel, S., Shrestha, A.K., Thapa, R. B., & Tiwari, S. (2021). Effects of different mulches and net house on crucifer aphid (*Brevicoryne brassicae* L.) population, growth and yield of broadleaf mustard (*Brassica juncea*). *Journal of Agriculture and Natural Resources*, *4*(2), 98-110. DOI: https://doi.org/10.3126/janr.v4i2.33677

INTRODUCTION

Broadleaf mustard, *Brassica juncea* var. Rugosa Bailey is a commonly grown leafy vegetable in Nepal, which is mainly grown during the winter season in the terai region and during the rainy season in the hilly region of the country. The broadleaf mustard was grown in 12,407 ha

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with a production of 153,602 mt and 12 mt/ha productivity (Ministry of Agriculture Development, 2017). Cabbage aphid (*Brevicorne brassicae* L.), mustard aphid (*Lipaphis erysimi* Kalt.) are the major insect pest of cruciferous crops throughout the world (Razaq *et al.,* 2011). The crucifer aphid, (*B. brassaicae*) is one of the important aphids prevalent in Asia causing significant crop loss in crucifers (Kumar & Singh, 2015). Most of the farmers rely on chemical pesticides to manage insect pests in different crops in Nepal (Rijal *et al.*, 2018; Regmi *et al.*, 2016). Famers are frequently using synthetic insecticides to control cabbage aphid and other prevalent insects of crucifers (Grzywacz *et al.*, 2010). Along with other alternative measures, the chemical method of pest control is unavoidable throughout the world (Razaq *et al.*, 2011). However, misuse of the chemical pesticide and chemical pollution is the major concern in the commercial agriculture pocket (Sharma *et al.*, 2012) and misuse of pesticides in vegetable pockets area lead to different serious health problems to the farmer in Nepal (Pujara & Khanal, 2002; Atreya, 2005). The excessive use of pesticides leads to loss of ecosystem resilience, biodiversity loss, pest resurgence, pest resistance, soil pollution, water pollution, bioaccumulation, biomagnification, air pollution, and food contamination and these entire problems have necessitated the use of alternative integrated measures to sustain the management of insect-pests (Atreya *et al.*, 2011). The problem associated with pesticide use can be overcome by growing crops in a protected environment such as net house and use of mulching materials. Growing crops inside the net house (Neupane *et al.*, 2018; Bhusal *et al.*, 2019) and use of mulching (Orozco-Santos *et al.*, 1995) lower insect pest population and increase yield. Therefore, this study was carried out to evaluate the efficacy of different mulching materials and net house against crucifer aphid of broadleaf mustard during the winter season in Chitwan, Nepal.

MATERIALS AND METHODS

Experimental Site

The field experiment was conducted at the Horticultural Farm, Agriculture and Forestry University (AFU) (www.afu.edu.np), Chitwan. It is located in the Terai region of Bagmati Province (27^0 37’ N latitude, and 84^0 25’ E longitudes with an elevation of 256 meter above sea level). The weather data during the research period was collected from nearby National Maize Research Program, Rampur, Chitwan, Nepal.

Table 1. Monthly weather data of Rampur, Chitwan, 2016

Month	Max. Temp. ^{0}C	Min. Temp. ^{0}C	Rainfall (mm)	Sunshine hours
September	32.32	24.88	21.04	4.16
October	31.81	21.25	1.37	6.89
November	28.57	13.71	0	6.80
December	23.80	10.15	0	4.85

Source: NMRP, Rampur, Chitwan

Experiment layout

Khumal broadleaf variety of broadleaf mustard was purchased from nearby agro-vet and seedlings were grown inside net house (16m×6m) in the tray using cocopits as a growing substrate. The experiment was laid out in Randomized Complete Block Design (RCBD) consisting of five treatments [T1 = untreated control, T2 = black plastic mulch only, T3 = reflective plastic mulch only, T4 = black plastic mulch with imidacloprid 70 WSG@0.13g/L,

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T5 = net house (40 mesh size) with black plastic mulch], which was replicated four times. The size of each plot was 1.5m × 2m (3m^2) and 50 cm space was maintained between plots. The seedlings of 25 days were transplanted with a spacing of 30cm×10cm in each plot maintaining 50 plants in each plot and 10 plants were marked as a sample for the recording of aphid number. The farmyard manure @6 t/ha and NPK @60:40:20 kg/ha were applied in the main field. The first spray was done at 25 days after transplanting in treatment black plastic mulch with imidacloprid 70WSG @0.13 g/L, at which a significant number of aphids were recorded in yellow sticky traps, and repeated at 15 days interval. Harvesting started at 32 days after transplanting by handpicking large harvestable leaves and eight harvestings was done during the entire research period.

Statistical analysis

The numbers of adult crucifer aphids were recorded from three large leaves of sample plants and the average number of aphids per plant was calculated. Numbers of aphid were recorded after 4, 8, 12 and 15 days of spray in insecticide-treated plot and numbers of aphid from other plots were also recorded on the same date. Plant height, number of effective leaves, plant canopy was recorded from ten sample plants at 10 DAT, 20 DAT and 30 DAT. The total yield from each treatment was calculated after harvesting the whole plot to calculate yield per hectare. The benefit-cost ratio of each treatment was calculated based on the prevailing market price of inputs and the price of vegetables. Results were statistically analyzed using ANOVA in R (R Windows 3.5.2) and mean were further separated at 5% significance level by Duncan Multiple Range Test (DMRT). Aphid population was analyzed after square root transformation (√x+0.5) as suggested by Gomez and Gomez (1984). Further yield comparison was done using the formula as follows:

Increase in yield over control (%) $= \left[\frac{(T-C)}{C}\right] \times 100$

Where, T= Marketable yield of the treated plot, and C= Marketable yield of control plot.
Means were compared by least significance difference (LSD) for treatment difference at 5%, 1% and 0.1% level of significance (Gomez & Gomez, 1984; Shrestha, 2019).

RESULTS

Effect of mulching and net house on crucifer aphid population

Four days after the first spray (DAS), aphid population was the lowest in net house + black plastic mulch (0.75) which was statistically similar with black plastic mulch + imidacloprid spray (3.00) and reflective plastic mulch (5.25). The untreated control had the highest aphid population (66.00) followed by black plastic mulch only (16.25) (Table 2). Similarly, 8 DAS, black plastic mulch + imidacloprid spray (1.50) and net house + black plastic mulch (2.00) had the lowest aphid population followed by reflective plastic mulch (7.50) but untreated control (63.50) had the highest aphid population followed by black plastic mulch (20.00) (Table 2). Likewise, at 12 DAS, net house + black plastic mulch (0.00) and black plastic mulch + imidacloprid spray (4.50) showed the lowest aphid population. The control plot (69.00) had the highest number of aphid followed by black plastic mulch (19.75) and reflective plastic mulch (13.50) (Table 2). Finally, at 15 DAS, net house + black plastic mulch (0.50) had the lowest aphid population which was statistically similar to black plastic mulch + imidacloprid spray (2.50). Aphid population was the highest in the control plot (73.50) followed by black plastic mulch (26.00) and reflective plastic mulch (14.50) plots (Table 2).

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Table 2. Effects of different mulching and net house on number of crucifer aphid (*B. brassicae)* per plant (square root transformation) on broadleaf mustard after the first spray at Rampur, Chitwan, 2016

Treatments	Number of aphid per plant			
	4DAS	8DAS	12DAS	15 DAS
Black plastic mulch only	4.08b (16.25)	4.51b (20.00)	4.47b (19.75)	5.10b (26.00)
Reflective plastic mulch only	2.37c (5.25)	2.82c (7.50)	3.73b (13.50)	3.79c (14.50)
Black plastic mulch+ Imidacloprid 70 WSG@0.13g/ L water	1.73c (3.00)	1.28d (1.50)	2.13c (4.50)	1.56d (2.50)
Net + Black plastic mulch	1.06c (0.75)	1.41cd (2.00)	0.71c (0.00)	0.93d (0.05)
Untreated Control	8.00a (66.00)	7.90a (63.50)	8.18a (69.00)	8.51a (73.50)
F test	***	***	***	***
SEM	0.50	0.44	0.52	0.23
CV (%)	29.13	26.18	26.64	16.87
P value	<0.001	<0.001	<0.001	<0.001
LSD(0.05)	1.55	1.45	1.58	1.03

****Significance at 0.001 level of significance, ns= Non-significant, CV: Coefficient of variation, Means followed by the same letter in each column are not significantly different at 0.05 level of probability, Value in parenthesis indicate back transformation, SEM: Standard error of means, P value: Probability value, LSD (0.05): Least significant difference at 0.05 level of probability*

Table 3. Effects of different mulching and net house on number of crucifer aphid (*B. brassicae*) per plant (square root transformation) on broadleaf mustard after the second spray at Rampur, Chitwan, 2016

Treatments	Number of aphid per plant			
	4 DAS	8 DAS	12 DAS	15 DAS
Black plastic mulch only	11.31b (128.00)	11.17b (127.25)	10.35b (112.50)	12.42b (155.50)
Reflective plastic mulch only	9.30b (93.00)	10.49b (116.75)	11.80b (140.00)	12.33b (151.50)
Black plastic mulch + Imidacloprid 70 WSG@0.13g/L water	1.44c (1.75)	1.53c (2.50)	2.14c (4.25)	2.21c (4.50)
Net + Black plastic mulch	0.71c (0.00)	1.49c (1.75)	1.22c (1.25)	1.26c (2.00)
Untreated Control	16.41a (269.50)	15.90a (253.75)	15.94a (256.00)	17.64a (311.75)
F test	***	***	***	***
SEM	1.02	0.92	0.82	0.60
CV (%)	18.26	16.72	15.48	11.98
P value	<0.001	<0.001	<0.001	<0.001
LSD(0.05)	2.20	2.09	1.98	1.69

****Significance at 0.001 level of significance, ns= Non-significant, CV: Coefficient of variation, Means followed by the same letter in each column are not significantly different at 0.05 level of probability, Value in parenthesis indicate back transformation, SEM: Standard error of means, P value: Probability value, LSD (0.05): Least significant difference at 0.05 level of probability*

The trend of aphid population after the second (Table 3) and the third spray (Table 4) was

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ISSN: 2661-6270 (Print), ISSN: 2661-6289 (Online)
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similar to that of the first spray. The maximum aphid population was recorded in untreated control whereas net house + black plastic mulch and black plastic mulch + imidacloprid spray had the lowest aphid population. Reflective plastic mulch had the lower aphid population compared to black plastic mulch and untreated control.

Table 4. Effects of different mulching and net house on number of crucifer aphid, *B. brassicae* per plant (square root transformation) on broadleaf mustard after the third spray at Rampur, Chitwan, 2016

Treatments	Number of aphids per plant			
	4 DAS	8 DAS	12 DAS	15 DAS
Black plastic mulch only	11.30b (128.00)	11.15b (127.25)	10.33b (112.50)	12.40 b (155.50)
Reflective plastic mulch only	9.27b (93.00)	10.47 b (116.75)	11.78b (140.00)	12.31b (151.50)
Black plastic mulch + Imidacloprid 70 WSG@0.13g/L water	1.21c (1.75)	1.24 c (2.50)	2.02c (4.25)	2.10c (4.50)
Net + Black plastic mulch	0.22c (0.00)	1.33 c (1.75)	0.91c (1.25)	0.88c (2.00)
Untreated Control	16.39a (269.50)	15.88a (253.75)	15.92a (256.00)	17.63a (311.75)
F test	***	***	***	***
SEM	1.07	0.94	0.81	0.66
CV (%)	19.04	17.08	15.56	12.67
P value	<0.001	<0.001	<0.001	<0.001
LSD(0.05)	2.25	2.11	1.96	1.77

****Significance at 0.001 level of significance, ns= Non-significant, CV: Coefficient of variation, Means followed by the same letter in each column are not significantly different at 0.05 level of probability, Value in parenthesis indicates back transformation, SEM: Standard error of means, P value: Probability value, LSD (0.05): Least significant difference at 0.05 level of probability*

Effect of different mulching and net house on yield and yield attributing characters of broad leaf mustard

Plant height

After ten days of transplanting, the highest plant height was observed inside the net house with black plastic mulch (16.58 cm). The lowest plant height was recorded in the untreated control (12.32) which was statistically similar to black plastic mulch (12.49 cm), black plastic mulch + imidacloprid spray (12.50 cm), reflective plastic mulch (13.28 cm). A Similar trend in plant height was recorded twenty days after transplanting. Again, at thirty days after transplanting, the highest plant height was recorded inside the net house (40.63cm) followed by reflective plastic mulch (33.39) and black plastic mulch + imidacloprid spray (33.34 cm). The lowest plant height was recorded in the untreated control (26.11 cm) followed by black plastic mulch (31.41cm) (Figure 1).

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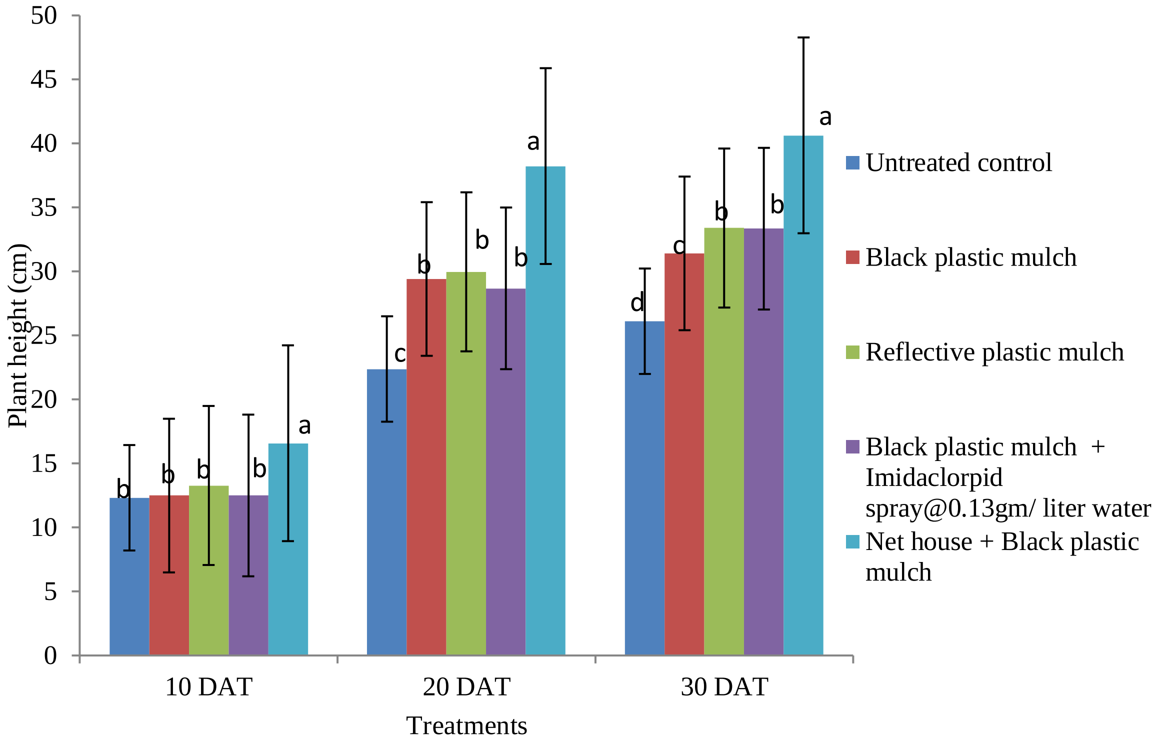

Figure 1. Effects of different mulching and net house on plant height of broadleaf mustard at Rampur, Chitwan, Nepal, 2016

Number of leaves

The number of leaves was statistically significant at 10, 20 and 30 DAT among different treatments. At 10 DAT, the number of leaves was the highest inside net house + black plastic mulch (5) followed by reflective mulch (5.25) and the lowest number of leaves was recorded in the untreated control (4.25) followed by black plastic mulch (5) and black plastic mulch + imidacloprid spray (5). Similarly, at 20 DAT, the highest number of leaves was recorded inside net house + black plastic mulch (10) which was statistically similar with black plastic mulch + imidacloprid spray (10) and black plastic mulch (9.75). The lowest number of leaves was recorded in the untreated control (8) followed by reflective mulch (9). At 30 DAT, the highest number of leaves was recorded inside net house + black plastic mulch (10.5) which was statistically similar with reflective plastic mulch (10.25), black plastic mulch + imidacloprid spray (10) and black plastic mulch (10) and the lowest number of leaves was recorded in the untreated control (9.25). However, the insecticide application was not done at 25 DAT in the black plastic + insecticide spray plot (Figure 2).

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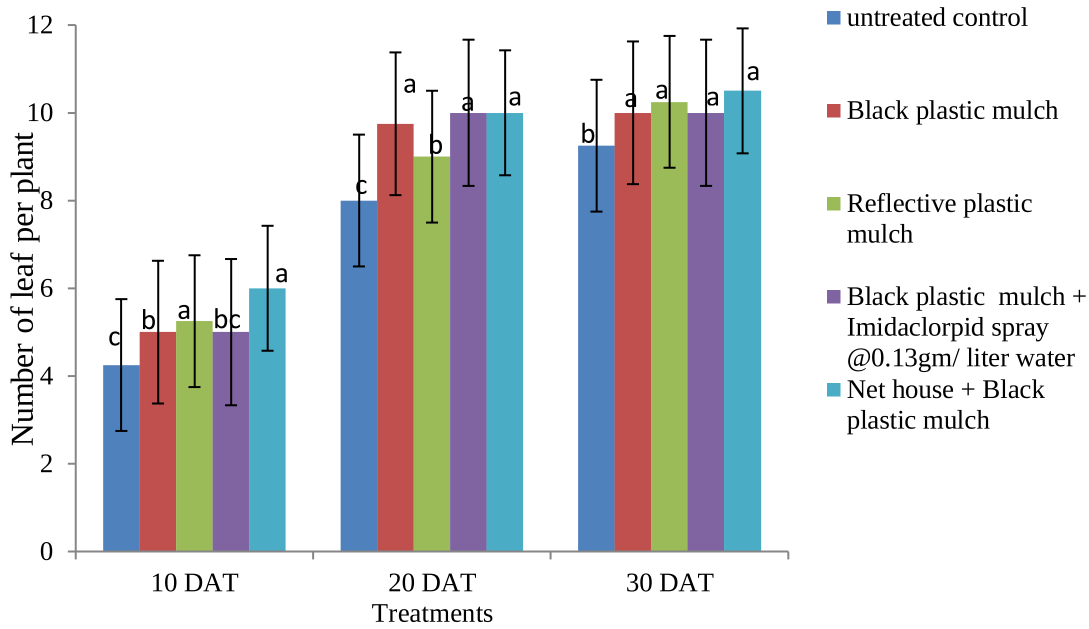

Figure 2. Effects of different mulching and net house on the number of leaves of broadleaf mustard at Rampur, Chitwan, Nepal, 2016

Plant canopy

At 10 DAT, the highest plant canopy was recorded inside net house + black plastic mulch (19.11 cm) followed by reflective plastic mulch (16.72 cm) whereas, the lowest canopy was noted in untreated control followed by black plastic mulch (16.09 cm) and black plastic mulch + imidacloprid spray (16.41 cm).

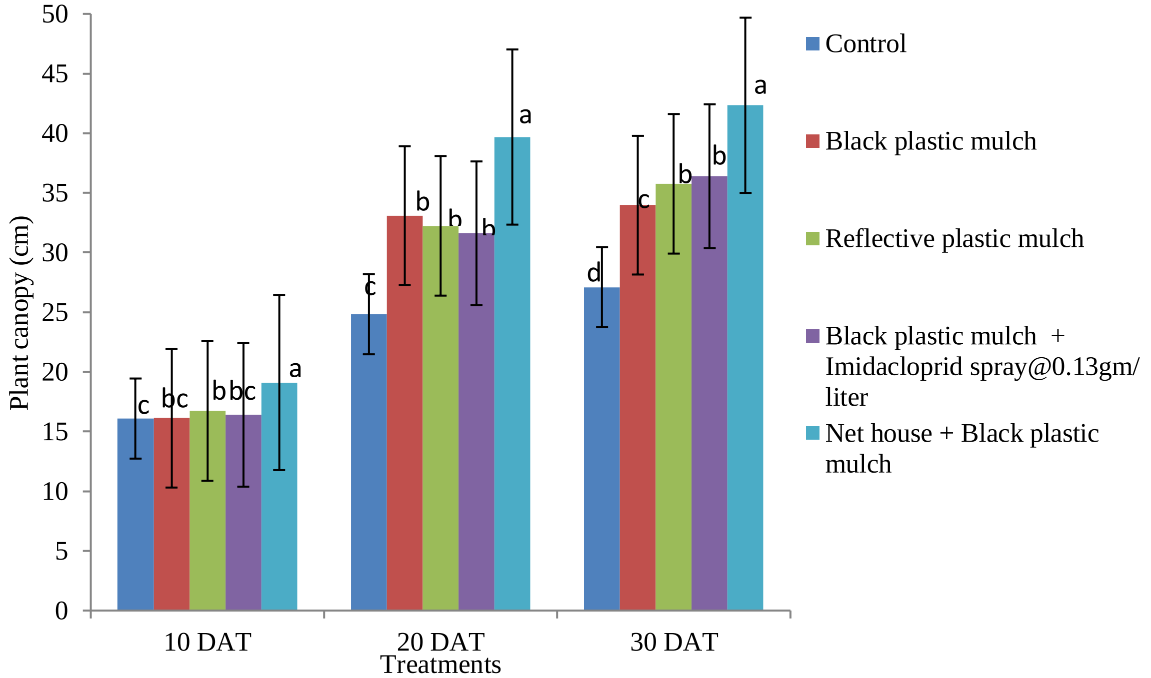

Figure 3. Effects of different mulching and net house on plant canopy of broadleaf mustard, Chitwan, 2016

ISSN: 2661-6270 (Print), ISSN: 2661-6289 (Online)
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A Similar trend on plant canopy was found at 20 DAT. At 30 DAT, the lowest canopy was recorded in the untreated control (27.10 cm) followed by black plastic mulch (33.97 cm); whereas, the highest plant canopy was recorded in net house + black plastic mulch (42.34 cm) followed by black plastic + imidacloprid spray (36.40 cm) and reflective plastic mulch (35.76 cm). The imidacloprid was applied at 25 DAT in the black plastic +imidacloprid spray plot (Figure 3).

Yield

The effects of different mulches and net house on yield of boardleaf mustard was given in Table **5.**

Table 5. Effects of different mulching and net house on the yield of broadleaf mustard and increase in yield over control at Rampur, Rampur, Chitwan, 2016

Treatments	Yield (t/ha)	Increase in yield over control (%)
Black plastic mulch only	20.35d	77.26
Reflective plastic mulch only	21.51c	87.37
Black plastic mulch + Imidacloprid 70 WSG@0.13g/L water	25.99b	126.39
Net house+ Black plastic mulch	26.86a	133.97
Untreated Control	11.48e	-
F test	***	
SEM	0.82	
P-value	<0.001	
CV (%)	1.90	
LSD(0.05)	0.63	

****Significance at 0.001 level of significance, ns= Non-significant, CV: Coefficient of variation, Means followed by the same letter in each column are not significantly different at 0.05 level of probability, SEM: Standard error of means, P value: Probability value, LSD (0.05): Least significant difference at 0.05 level of probability*

The yield of fresh leaves of broadleaf mustard was the lowest in the untreated control (11.48 t/ha) followed by black plastic mulch (20.35 t/ha). The highest yield was obtained from net house + black plastic mulch (26.86 t/ha) followed by black plastic + imidacloprid spray (25.99 t/ha) and reflective plastic mulch only (21.51 t/ha) (Table 5). Therefore, the maximum increase in yield over control (%) was obtained in net house + black plastic mulch (133.97%) and black plastic much+imidacloprid spray (126.39 %) and minimum increase in yield over control (%) obtained with black plastic mulch (77.26 %) followed by reflective plastic mulch (87.38%) (Table 5).

Effect of different mulching and net house on economic analysis

The maximum benefit-cost ratio was obtained from black plastic mulch + imidacloprid spray (4.09) followed by reflective plastic mulch (3.42), black plastic mulch (3.32), net house+ black plastic mulch (3.10) and the minimum benefit-cost ratio was obtained in control plot (2.30) (Table 6).

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Table 6. Economic analysis of broadleaf mustard as affected by different mulching treatments at Rampur, Chitwan, Nepal, 2016

Treatments	Cost of cultivation (US$ /ha)	Gross return (US$)	Net return (US$)	B:C ratio
Black plastic mulch only	2026.15	6726.16	4700.01	3.32
Reflective plastic mulch only	2080.51	7108.25	5027.73	3.42
Black plastic mulch + Imidacloprid 70WSG@0.13g/L water	2099.56	8590.17	6490.62	4.09
Net + Black plastic mulch	3575.74	11099.46	7523.72	3.10
Untreated Control	1652.07	3792.71	2140.65	2.30

* Market price: US$ 0.58 and US$ 0.29 per kg fresh broadleaf mustard for October and November 2016 respectively.

* Net house and other input costs on the prevailing market price

DISCUSSION

Effects of different mulching and net house on aphid population

During the field experiment, the population of crucifer aphid, *B. brassicae* was significantly different among different treatments. The net house with black plastic mulch almost restricted the aphid population. The black plastic mulch only did not reduce the aphid population so it is sure that net house work as a physical barrier against insect inside it and black mulching obstructed the growth of weed. Boiteau and Vernon (2004) reported that net house created physical barriers between plants and insects so reducing insect pest populations inside it. Insect nets enable passive control of flying insect pests of vegetable crops, thus reducing the pesticide sprays (Martin *et al*., 2006). Similarly, insect nets also have indirect impacts on the behaviour of insect pests by modifying the visual and olfactory signals on nets mask the crop (Mazzi & Dorn, 2012), thus deterring insect pests that detect their prey via visual signals (Weintraub & Berlinger, 2004). The aphid population was slightly higher in black plastic mulch + imidacloprid spray than in net house + black plastic mulch but these two treatments are statistically similar in almost cases. Imidacloprid is an effective systemic insecticide (Nauen *et al*., 1998) with a high degree of residual activity against aphids although the compound is slow acting (Boiteau & Osborn, 1997). According to Gervais *et al*. (2010) imidacloprid disrupts the nerve's ability to send a normal signal and the nervous system of insect stops working so the insect feeding it may die. Next to insecticide spray, reflective plastic mulch reduces the number of crucifer aphid significantly as compared to black plastic mulch. Zalom (2017) also reported reflective mulches superior to black plastic mulch, as these mulches repelled aphids from them (Adlerz & Everett, 1968). Brown *et al*. (1993) reported that silver plastic mulch superior to white, yellow, or black plastic mulch in summer squash. Schalk and Robbins (1987) also found that aphids were repelled by silver coloured mulch.

Effects of different mulching and net house on yield and yield attributing characters of boardleaf mustard

The first insecticide spray was done only at 25 DAT in black plastic + imidacloprid spray treatment. The plant height, leaf number and canopy were the highest inside the net house + black plastic mulch followed by black plastic mulch + imidacloprid spray, reflective plastic mulch and black plastic mulch. The lowest plant height, leaf number and canopy were recorded in the untreated control. The net house had the shading effect and mulching suppresses the

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weed and reduces the moisture loss in the soil so net house + black mulching had the superior result compared to other treatment. This vegetative parameter was superior in reflective mulch compared to black plastic mulch as reflective mulch has repellent characteristics against aphid and other small insects. Neupane *et al.* (2018) also reported the highest yield attributing characteristics in broadleaf mustard inside net house + black plastic mulch followed by black plastic mulch + imidacloprid spray, reflective mulch and black plastic mulch.

The highest yield was obtained in net house + black plastic mulch followed by black plastic mulch + imidacloprid spray. The net acts as a physical barrier to insect pest whereas black mulching suppress the weed so the maximum yield was obtained. Plants on the net house plots grew more vigorously with a higher number of leaves, leaf area and leaf spreading characters. A fine mesh improves the netting efficacy against pests and also had a major impact on the microclimate. In sub-tropical climatic conditions, the netting efficacy and beneficial microclimate improved crop yields (Simon *et al.*, 2014). Furthermore, mulching affected insect pest and improve yield attributing characters such as plant height, canopy, leaf number because it prevents weed and washing of soil nutrient during heavy rains. The soil under plastic mulch remains loose, friable, well-aerated, and have access to adequate oxygen and microbial activity. Mohanty (1977) reported a similar result with the use of mulching materials. This can be also supported by the chimney effect; plastic mulch is impervious to carbon dioxide gas which releases through a hole made in plastic for plant growth resulting in localized concentrations of abundant CO_2 for the actively growing leaves which increased the yield of turmeric (Mukulkumar *et al.*, 2018). Imidacloprid is an effective systemic insecticide (Nauen *et al.*, 1998) with a high degree of residual activity against aphids so plant productivity is enhanced as compared to control. Similarly, the yield was higher in reflective mulch as compared to black plastic mulch and untreated control. The plastic mulches resulted in an 84-98% reduction in weed biomass which directly influences crop yield (Rajablariani *et al.,* 2012). Mulches prevent soil water evaporation retaining soil moisture (Ramakrishna *et al.,* 2006). Sometimes black plastic mulches can create soil temperatures that are too high and this will cause deleterious effects to plant growth (Diaz-Perez *et al.*, 2000). Brown *et al*. (1993) found reflective plastic mulch was superior to white, yellow or black plastic mulch for repelling aphids so yield is higher in reflective mulch as compared to other mulch.

Effects of different mulching and net house on economic analysis

There is a lower cost of cultivation and higher economic benefit in black plastic mulch with imidacloprid spray. Even there is higher production in net house with black plastic mulch than average but its cost of cultivation is quite higher. However, in the long run, use of net house with mulching can be the ecofriendly viable option for broadleaf mustard production. Use of net house not only improves crop performance but also minimize the number of insecticide sprays within the crop cycle, which could lead to less rejection of produce in the export market, improved environmental quality and economical in long run (Gogo *et al.,* 2014). The net house also reduced the number of pesticide applications by 90% which reduces the cost of chemicals spraying (Majumdar & Powell, 2011) thus producing pesticide-free products.

CONCLUSION

Crucifer aphid, *B. brassicae,* is one of the major insect pests of broadleaf mustard causing significant loss to fresh vegetable production. The net house plus black plastic mulch and black plastic mulch plus imidacloprid spray were effective to prevent aphid population resulting in better plant growth and higher yield in broadleaf mustard. The highest benefit-cost ratio was achieved with the use of black plastic mulch with imidacloprid spray. However, net house + black plastic mulch is a good method of insect pest management as it limits insecticides application. Thus, the use of net houses along with mulching is proven to be favorable in the long run for higher quality production and healthier production which is an important tool for vegetable growers. However, use of disposable plastic or plastic recycling is suggested to reduce a plastic pollution in future.

ACKNOWLEDGEMENTS

The authors are grateful to Asia Innovative Farmers Activity (AIFA) Project of WINROCK International for funding this research. We also thank the Directorate of Research and Extension, AFU, Rampur, Chitwan for their support during the research period.

Authors contributions

RR and AKS developed the project. RR drafted the paper and RBT and ST revised the paper. SP collected data and analyzed

Conflict of interest

The authors declare no conflicts of interest regarding publication of this manuscript.

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ISSN: 2661-6270 (Print), ISSN: 2661-6289 (Online)
DOI: https://doi.org/10.3126/janr.v4i2.33677

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