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RESEARCH ARTICLE

Microbial consortium: an eco-friendly approach against *Alternaria brassicae* in Indian mustard

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Abstract

Indian mustard is the third largest oilseed crop and important for the food security concern. Among various diseases of mustard crop, Alternaria leaf spot also known as Alternaria blight, causes yield loss of up to 70%. The use of chemicals to treat the diseases is not environmentally friendly, lead poor health of soil and damage food for the consumption. The combinations of multiple antagonistic organisms may provide improved disease control over the use of single organisms. Multiple organisms enhance the level and consistency of control by providing multiple mechanisms of action; combinations of fungi and bacteria may provide protection at different times or under different conditions and complementary niches. This investigation was performed to explore the potential of consortium of rhizospheric bacteria and fungi to use them as bio control agents for suppression of the blight of mustard and plant growth-promoting activities. The inoculated seeds were established under greenhouse and field conditions. Based on the results, one out of four consortia has shown reduction of disease incidence by 28% and increase in seed yield by 42% as compared to control under field conditions against the pathogen. The growth parameters like length of the leaves, roots, stem, plant height, numbers of leaves, and seed development were measured after 8 weeks of planting. Microbial consortia increased the growth parameters better in comparison to single inoculant treatments. Thus, the consortia could be a reliable alternative instead of chemical fertilizers and pesticides for mustard.

Keywords Chemical fertilizer · Eco-friendly · Microbial consortium · PGPR · *Trichoderma*

Introduction

The diverse community of microbes on earth has a vital role in the environment's geological and biological processes (Tringe et al. 2005; Xu 2006). Biocontrol agents, including fungi and bacteria, have distinct mechanisms against plant pathogens. Fungal biocontrol agents act against the pathogen through physical contact, while bacterial biocontrol is based on antibiosis mechanism for disease suppression (Howell 2003; Mohiddin et al. 2010). Application of microbial biofertilizers in tomato increases the soil properties and act as compatible fertilizer (Mpanga et al. 2018) and amalgam of two or more beneficial microbes increase the efficiency of disease suppression with biocontrol formulations (Haggag and Nofal 2006).

Combination of two or more different species of microorganisms that work together as a community is termed as microbial consortium, some of the previous work reported on the combination of microbial inoculants includes a consortium of bacteria (Raupach and Klopper 1998), consortium of biocontrol fungal and bacterial isolates (Shah et al. 2008). Development of consortium requires microorganisms which are easy to handle, long shelf life, non-pathogenic, have synergistic effect, have natural defence system against pathogens, and are compatible with each other. Combination of biocontrol agents improved the growth (Kumar et al. 2016a; Kumar et al. 2016b; Berendesen et al. 2018). In the last few years, stress is shifted towards the utilization of biocontrol agents (BCAs) with distinct mechanisms against soil-borne pathogens and to increase their efficiency against numerous diseases. Literature supports that many species of *Trichoderma* and *Pseudomonas* have great biocontrol potential against fungal pathogens (Zegeye et al. 2011; Bhattacharjee and Dey 2014; Sood et al. 2020) and microbial

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consortia of *Pseudomonas* and *Trichoderma* either though seed treatment or foliar spray in the soil successfully reduce the disease intensity of the crop (Manjula et al. 2004). An approach involving Plant Growth Prompting Rhizobacteria (PGPR) and its combination with different species and genera shows synergistic effect that has been applied (Beneduzi et al. 2012; Gouda et al. 2018).

Several reports have suggested that a combination of *Pseudomonas fluorescens* with several BCAs used for controlling plant diseases. The PGPR consortia showed significant increase in germination percentage and enhanced multiple plant growth characteristics, against *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum* (Syed et al. 2020). Microbial consortium of efficient strains for biological control helps in improving microbial efficacy, reliability, and consistency under diverse soil and environmental conditions (Sharma et al. 2020), and will work against a wide range of pathogens and would also improve the quality of the crop (Chaube and Sharma 2002).

The objective of the present study is to evaluate the efficacy of microbial consortia of selected biocontrol agents to reduce Alternaria blight disease and to check the variation in growth parameters in *Brassica juncea* caused by *A. brassicae* under laboratory, greenhouse, and field conditions.

Materials and methods

Isolation of biocontrol agents and fungal pathogen

Isolates were collected from mustard fields at Indian Agricultural Research Institute (IARI), Delhi (Latitude 28°38′23"N, Longitude: 77°09′27"E., Altitude: 228.61 m above sea level). The bacterial isolates were maintained on nutrient agar slants while fungal isolates on PDA agar slants at 4 °C during the study. In the present investigation, two *Trichoderma harzianum* isolates designated as Th3 (Accession no. MW041160) and Th2 (Accession no. MW041161) and two *Pseudomonas* isolates designated as B12 (Accession number MT704967) and B3 (Accession number MT704966) (Gupta et al. 2020) were chosen due to their antagonistic effect against *Alternaria brassicae*.

Infected leaves with characteristic symptoms like necrosis, concentric black rings were picked and incised into 5–10 mm bits, then were sterilized using 0.1% sodium hypochlorite solution followed by washing with sterile distilled water twice and air dried for few minutes. The small leaf bits were further inoculated on Potato Dextrose Agar (PDA) media plates and were incubated in BOD for 3–5 days at temperature of 25 ± 2 °C followed by their morphological and microscopic identification. They were identified as *Alternaria brassicae* based on their mycelial growth, colony characteristics and microscopic examination.

In vitro compatibility test

Compatibility tests of all the four selected BCAs were performed on PDA media using the method described by Manjula et al. (2004).The fungal bio control agents were classified into two groups: slow and fast growing fungi. The fungi covered the full plate within 3 days and those needed more than 3 days were considered as fast growing and slow growing fungi, respectively (Upamanya et al. 2020).For this experiment, the fungal and bacterial bio control agents were inoculated simultaneously as both the BCAs were fast growing. Lack of inhibition zone at the point of intersection indicates the compatibility between two strains (Jha et al. 2012). All the experiments were repeated thrice. Ten combinations of microbes were tested for compatibility viz. B3 + B12, Th2 + Th3, B3 + Th2, B3 + Th3, B12 + Th2, B12 + Th3, B3 + B12 + Th2, B3 + B12 + Th3, Th2 + Th3 + B3 and Th2 + Th3 + B12.

Seed priming

Based on results obtained in compatibility experiments, microbial consortia were prepared using the efficient and compatible isolates of *Pseudomonas* and *Trichoderma*. Mustard seeds (var. Pusa Vijay) used in the experiment were sterilized by soaking them in a solution of sodium hypochlorite solution (0.5%) for 2–3 min and seeds were further rinsed with distilled water. For mustard seeds priming talcum powder was used as a carrier with an inoculum of *Pseudomonas* and *Trichoderma* in combination then slurry was prepared using 10 g of *Trichoderma* formulation, and 100 ml *Pseudomonas* broth and the cfu count of the culture was maintained at 2×10^5 mL^{-1}. Sterilized mustard seeds were then drenched in this slurry overnight at room temperature then seeds were transferred to the blotting sheet for air drying. Seeds treated with 1% carboxy methyl cellulose (CMC) served as control.

Bioefficacy of microbial consortia against *A. brassicae* under pot and field conditions

The experiments were performed to check the ability of microbial consortia under greenhouse and field conditions using bio primed seeds. The treatment combinations with 3 replications were as follows:T 1 = Control (Pathogen *i.e. A. brassicae*), T 2 = B3 + Th2 + *A. brassicae,* T 3 = B3 + Th3 + *A. brassicae,* T 4 = B12 + Th2 + *A. brassicae* and T 5 = B12 + Th3 + *A. brassicae.*

Effect of microbial consortium on germination of mustard seed under *in vitro* conditions

Ten bio-primed seeds of mustard of each treatment were placed on water agar plates to check their compatibility

and rate of germination. The experiment was performed in replicates of three plates of each treatment. The seeds were treated with CMC and *A. brassicae* served as control. Regular monitoring was conducted, and the data recorded after 10 days.

Pot and field trials of microbial consortium

To evaluate the efficacy of developed consortia against *Alternaria* blight in Indian mustard under greenhouse conditions, plants were grown in earthen pots of 30 inches depth and 20 inches diameter containing soil, three pots per treatment were used as replicates (Abd-El-Khair et al. 2019). Ten bio primed seeds per pot were sownat 2 cm deep to check the germination rate. Thinning was performed after 30 days, and only five plants per pot were evaluated. Irrigation was provided once in a week.

The field experiment was performed during two consecutive years 2016–2017 and 2017–2018 from October to April at Department of Plant Pathology, Indian Agriculture Research Institute (IARI), New Delhi (Latitude 28°38′2″N, Longitude: 77° 09′27″E, Altitude: 228.61 m above sea level) in completely randomized block design (CRD) using four replications of mustard plants in each row with two-row replications of each treatment. The spacing of 25 cm seed to seed and spacing of 65 × cm from row to row was maintained. All the plant biometric parameters like seed germination, plant height, and seed yield were evaluated up to 90 days of sowing or germination.

The germination percentage and seed vigour index was calculated as described by Abdul and Anderson (1973).Germination percent, seed vigour was calculated according to Jha et al. (2012). Disease incidence was recorded at 15 days post inoculation (dpi) of the pathogen and was measured disease severity using scale 0–5 (Shrestha et al. 2005), where 0 indicates no infection, 1 = 1–10% leaf area covered by the disease, 2 = 10–20% leaf area covered, 3 = 20–30% leaf area covered, 4 = 30–40% leaf area covered, 5 = 40–100% leaf area covered. Observations were recorded and data was collected for the plant biometric parameters and seed production after 90 days of sowing.

Statistical analysis

All in vitro investigations were performed with three replicates for each treatment. The experiment for green house and field assay were performed in a completely randomized block system (CRD). Data were collected and statistically analysed with One-way ANOVA tests using SPSS software version 11.0. Mean data were compared with the significant difference at $P \leq 0.05$.

Results and discussions

In vitro compatibility test

The results of compatibility test revealed that two different species of microbes were growing well in the same plate without any inference while isolates of the same species did not show compatibility with each other. It was witnessed that out of all the consortia formulation applied, only few shows synergistic effect in consortia while others showed competition and restricted the growth of other microbe in presence of one. It was also observed that when all the four biocontrol agents were grown together in same plate, they did not show any compatibility and their mycelial growth was affected in the presence of other microbes. This may be due to competition of nutrients among the same set of species during growth. Bacterial isolates B3 and B12 when grown in individual treatment with only one fungal biocontrol agent have no effect on the growth of Th2 and Th3 (Fig. 1). There was no zone of inhibition, and lack of inhibition zone between the isolates, suggests that their antagonistic metabolites do not inhibit each other. Based on the compatibility test, all four isolates and their mixed combinations were selected for further studies.

Application of biocontrol consortia against fungal and bacterial pathogens can be utilized to the fullest by selecting most potential strains and not by arbitrary use of consortia (Sharma et al. 2020). Similar results have been observed in compatibility assay with, *P. fluorescens* GB 10 showing no effect on the growth of *T. viride*pq 1 and both showing synergistic effect with each other (Manjula et al. 2004). The radial growth of *T. harzianum*was favoured by the presence of *P. fluorescens* (Dandur and and Knudsen 1993). Harshita et al. (2019) also supported this study who reported compatibility of *T. harzianum* and *P. fluorescens* in both in vivo and in vitro conditions.

In vitro analysis of the microbial consortium

In this study, all the developed consortia were analysed through seed treatment in water agar plates by germination percentage evaluation (Fig. 2). Elastic increase was recorded in germination percentage in the treatments inoculated with

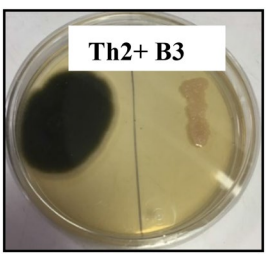

Fig. 1 In vitro compatibility test among biocontrol isolates

microbial consortia. Maximum germination (80%) was observed in T 2 (B3 + Th2 + *A. brassicae*) whereas minimum germination (68%) was recorded in T5 (B12 + Th3 + *A. brassicae*) compared to 55% in negative control without any treatment (Table 1). Based on the results of the above experiment, it is evident that seeds treated with consortium shows the increased germination and can further be evaluated under greenhouse and field conditions.

Table 1 Effect of different combination of biocontrol agents on seed germination of mustard in in vitro conditions

Treatment	% Germination
T1 (Control)	55
T2	80
T3	72
T4	70
T5	68
CD	1.305
SE (m)	0.394

T 1 = Control (Pathogen *i.e.A. brassicae*), T 2 = B3 + Th2 + *A. brassicae,* T 3 = B3 + Th3 + *A. brassicae,* T 4 = B12 + Th2 + *A. brassicae* and T 5 = B12 + Th3 + *A. brassicae*

Biocontrol assay of microbial consortium under greenhouse conditions

Soil-borne pathogen affects the germination of *B. juncea* crop by affecting its root system. All the developed consortia were evaluated under greenhouse conditions. Highest germination rate (75%) was observed in treatment T2 followed by T3 (70.00%), T4 (65.00%) and T5 (70.00%) respectively. The untreated seeds in treatment T1 (control) lowest germination of 45.00%. The outcome of this experiment reveals that the maximum vigour index (17,966.88) was recorded in T2, and the least vigour index (11,321.19) was recorded in T 5 (Table 2). The pooled data analysis (Table 2) indicated that application of consortia B3 + Th2 (T2) showed disease incidence of 45.3% as compared to control (69.7%), which was found significantly superior over all the treatments. All the treatments showed significant improvement in plant length and weight in comparison to the control. Maximum root length (33.53 cm), shoot length (147.00 cm), root fresh weight (25.69 g), shoot fresh weight (132.75 g), root dry weight (15.14 g), shoot dry weight (84.25 g) were observed in T2 and this was followed by T3 and T4 (Table 2).

Preliminary studies have underlined the importance of biocontrol agents like *Pseudomonas* spp. and *Trichoderma* spp. in reducing the intensity of foliar diseases of mustard crop (Jackson and Kumar 2019). The results are in accordance with several other workers who used combinations of biocontrol agents for the management of soil-borne disease (Mwangi et al. 2011). In a similar study on the tomato plant conducted by Singh et al. (2013), a combination of *T. harzianum* and *P. fluorescens* increased the growth of the plant and reduced the incidence of blight disease by 52.23% caused by *Alternaria solani.* The amalgam of potential biocontrol agents *Trichoderma* spp. and *P. fluorescens* gave better results in diminishing disease caused by *Ralstonia* spp. as compared to when applied as single biocontrol (Yendyo et al. 2017). Most of the research on the management of disease through biological control mostly focuses on a single strain, but the research nowadays has been shifted towards mixing two or more fungal or bacterial species together to attain better results (Vorholtet al. 2017; Woo and Pepe 2018). This is because mixed cultures can adapt to different environmental conditions and will have a dual-mode mechanism of action against the pathogen (Garcia et al. 2003; Sarma et al. 2015) (Fig. 3).

Higher seed yield was also witnessed in plants treated with the microbial consortium, compared to untreated control under greenhouse conditions (Table 2). Plants treated

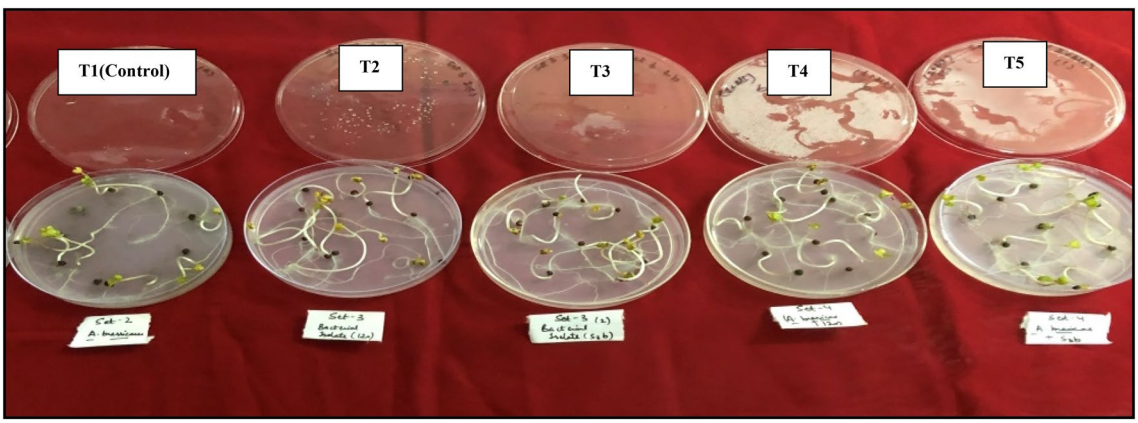

Fig. 2 In vitro analysis of the microbial consortium on seed germination of mustard after 10 days

Table 2 Effect of consortia on biometric parameters and seed production of *Brassica juncea* under greenhouse conditions

Treatment	% Disease incidence	% Germination	Root Length (cm)	Shoot Length (cm)	Fresh wt. Root (g)	Fresh wt. shoot (g)	Dry wt. root (g)	Dry wt. shoot (g)	Vigour index	Number of seed pods/plant	Seeds/seed pod	Total seed count/plant
T1 (Control)	69.7	45	19.94	117.88	20.13	118.81	9.75	55.00	9237.81	102.50	9.81	1608
T2	45.3	75	33.53	147.00	25.69	132.75	15.14	84.25	17,966.88	150.69	14.88	2245
T3	54.2	70	30.44	125.88	24.25	126.06	13.44	71.38	13,152.29	128.56	13.44	1729
T4	58.5	65	20.63	124.06	23.81	130.31	11.88	65.50	11,973.00	122.81	12.25	1501
T5	62	70	29.27	123.44	20.25	120.31	12.10	68.94	11,321.19	119.38	12.00	1427
CD	2.950	2.850	0.654	2.983	2.603	2.179	1.753	2.020	10.485	1.541	1.744	1.896
SE (m)	0.891	0.860	0.198	0.901	0.786	0.658	0.529	0.613	3.166	0.465	0.526	0.573

T 1 = Control (Pathogen *i.e. A. brassicae*), T 2 = B3 + Th2 + *A. brassicae*, T 3 = B3 + Th3 + *A. brassicae*, T 4 = B12 + Th2 + *A. brassicae* and T 5 = B12 + Th3 + *A. brassicae*

(a) (b)

Fig. 3 **a** Diseased mustard leaves in untreated control (T1), **b** Plants in Treatment T 2 (B3 + Th2)

with microbial consortia of *T. harzianum, P. aeruginosa* and *B. subtilis* enhanced seed yield and plant growth when applied in pigeon pea plant with root rot disease caused by *Sclerotinia sclerotiorum* (Jain et al. 2015).

Biocontrol assay of microbial consortium under field conditions

The results in Fig. 4 reveals that highest germination percentage of 84.5% was recorded in T 2, and the minimum was recorded in T1 (55.0%) which is control during first crop cycle whereas highest germination percentage of 80.0% was recorded in T4 during second crop cycle as compared to 47.5% in control (T1). Inoculation with microbial consortia (dual biocontrol) increases the plant growth and results a good yield of the plants. The lowest disease incidence of 45.6% was recorded in T4 compared to 74.5% in T1 (control) during first crop cycle. During second crop cycle T2 reported lowest disease incidence of 48.0% in contrast with T1 (68.5%).

Steady increase in all the plant growth parameters was also witnessed in both the crop cycles (2016–2017, 2017–2018). Plants treated with consortia B3 + Th2 (T2) shows maximum elevation in root length (42.0 cm,47.38 cm) and shoot length (190.75 cm, 206 cm) after 90 days of sowing, in compare to control root length (30.62 cm,30.38 cm) and shoot length (160.75 cm,162.50 cm) during both crop cycles (Table 3). A subsequent enhancement in root length is due to the increased uptake of nutrients by the plant.

The maximum increase in the fresh root and shoot weight of the plant was witnessed in T2 (87.50 g, 343.63 g), followed by T3 (68.75 g, 305.25 g), T4 (61.50 g, 327.63 g), and T5 (64.75 g, 310.63 g), compared to control (53.38 g, 208.13 g) during first crop cycles. Comparable results were witnessed in second crop cycle having maximum fresh weight in T 2 (98.75 g, 344 g) compared to control T 1 (55.38 g, 216 g). The highest increase in

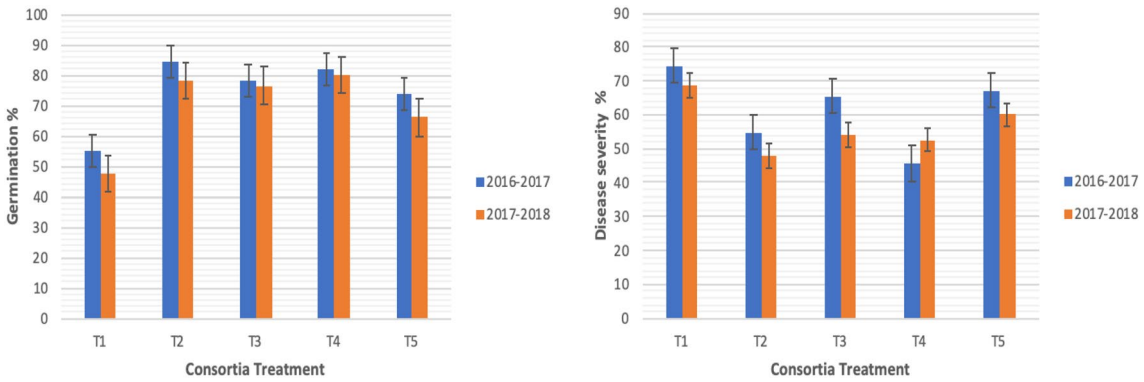

Fig. 4 Effect of consortia on seed germination (%) and disease severity (%) of *B. juncea* in field trials during both cropping years

dry root weight (46.50 g, 50.63 g) and dry shoot weight (186.25 g, 201.63 g) was also recorded inT2 followed by all the remaining treatments during both the crop cycles as displayed in Table 3).

Not much research has been conducted for the management of blight disease of mustard through the combined effect of *Trichoderma* spp. and *Pseudomonas* spp., but only individual effect against the disease has been reported. Integrated management of Alternaria blight and white rust in mustard by using a combination of *T. harzianum*, *Pseudomonas fluorescens*, different plant extracts, and fungicides has been reported by Rathi and Singh (2009).The microbial consortium of potential and effective biocontrol agents acts against a broad range of phytopathogens through multiple defense systems like synergistic effect and increasing plant resistance towards different pathogens (Singh 2016).The application of *T. viride*, along with neem and eucalyptus oil, significantly reduced the disease incidence of *A. brassicae* in mustard crops (Ansari et al. 2017). The results are in proximity with Istifadahet al. (2019), who reported a positive effect of microbial consortia against late blight of potato.

Results of seed production of T 2 (6072, 6436) have displayed an elevated increase compared to other treatments tested during two subsequent crop cycles (Table 3). All the biometric, vegetative parameters, and seed production tested, were higher in treatment 2 (B3 + Th2), the formulated consortia. Negligible disease incidence (include the disease incidence per cent) was observed in this treatment in field conditions. Treatment of plants with (B3 + Th2) was the most effective treatment among all the consortia tested in terms of plant growth and seed production The result of the above research corroborates with the findings of Kabdwal et al. (2019), who reported that integrated management with soil application of both *T. harzianum* and *P. fluorescens* increased the plant growth, yield and reduce the plant mortality in experimental fields.

Conclusion

Microbial consortia with four potential biocontrol agents viz, *Trichoderma* spp. and *Pseudomonas* spp. were studied for their combined effect against blight disease of mustard. The developed microbial consortia were found to have biocontrol ability and were highly compatible with each other. Results concluded that among four treatments, T2 with consortia of B3 and Th2 molecularly characterized as *P. fluorescens* and *T. harzianum* respectively was found to be the most effective consortia under greenhouse and field conditions. The outcome of the present work suggests that the combination of microbes has good potential in reducing the disease incidenceand helps in plant growth promotion with increased seed productivity.

Table 3 Effect of consortia on biometric parameters and seed production of *Brassica juncea* under field conditions during 1st crop cycle (2016–17) and 2nd crop cycle (2017–18)

Treatment	Year	Root length (cm)	Shoot length (cm)	Fresh wt. root (g)	Fresh wt. shoot (g)	Dry wt. root (g)	Dry wt. shoot (g)	Total seed count	Total no. of seed pods	Seeds per seed pod
T1 (Control)	2016–2017	30.62	160.75	53.38	208.13	19.56	109.75	3364	229	10
	2017–2018	30.38	162.50	55.38	216.00	20.89	118.13	4236	284	11
T2	2016–2017	42.00	190.75	87.50	347.63	46.50	186.25	6072	409	15
	2017–2018	47.38	206.00	98.75	344.63	50.63	201.63	6436	402	16
T3	2016–2017	39.62	187.00	68.75	305.25	39.75	177.38	5507	416	13
	2017–2018	38.25	187.13	66.63	306.75	41.38	197.13	5960	408	15
T4	2016–2017	30.87	187.37	61.50	327.63	25.88	112.25	5008	347	14
	2017–2018	32.38	197.25	60.88	325.50	26.63	127.25	5251	359	14
T5	2016–2017	30.62	182.37	64.75	310.63	32.63	121.88	5258	386	14
	2017–2018	31.50	189.75	68.75	324.75	37.75	139.00	5564	401	13
CD (2016–2017)		2.762	2.512	2.861	1.498	4.032	2.584	45.086	23.098	N/A
SE (m) (2016–2017)		0.834	0.758	0.864	0.452	1.218	0.780	13.614	6.97	1.576
CD (2017–2018)		2.549	6.259	2.565	2.796	2.176	2.999	65.233	29.598	N/A
SE (m) (2017–2018)		0.770	1.890	0.774	0.844	0.657	0.905	19.699	8.931	0.952

T 1 = Control (Pathogen *i.e. A. brassicae*), T 2 = B3 + Th2 + *A. brassicae,* T 3 = B3 + Th3 + *A. brassicae,* T 4 = B12 + Th2 + *A. brassicae* and T 5 = B12 + Th3 + *A. brassicae*

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