# *Trichoderma Asperellum* as A Promising Mycofungicide for Managing The Dieback Disease of Tea (*Camellia Sinensis*)

**Kishor Chand Kumhar** ( kishorkumarc786@gmail.com )
Chaudhary Charan Singh Haryana Agricultural University

**Azariah Babu**
Tea Research Association

**John Peter Arulmarianathan**
Varsha Bioscience India Private Limited

**Bhabesh Deka**
Tea Research Association

**Mitali Bordoloi**
Tea Research Association

**Hirakjyoti Rajbongshi**
Tea Research Association

**Pritam Dey**
Tea Research Association

**Research article**

**Keywords:** Trichoderma asperellum 2% AS, tea, dieback, yield, non-target organisms, phytotoxicity

**Posted Date:** September 3rd, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-58156/v1

# Abstract

**Background:** The dieback disease of tea caused by *Fusariumsolani* adversely affects its production and quality. Genus *Trichoderma* is a promising biocontrol agent to control it without any residual effect and most suitable for inclusion into an integrated disease management approach. Isolation of *Trichoderma* from rhizosphere soil of the Dooars zone was done. It was identified as *T. asperellum* based on its cultural characters and DNA fingerprinting. A liquid formulation (2% Aqueous Suspension) was prepared from it and bio-efficacy was evaluated under field conditions for the control of dieback disease and other parameters for two seasons in three zones. Its different concentrations, *T. harzianum,* and Hexaconazole were included in the study.

**Results:** The fungal isolate (KBN-29), identified as *T. asperellum,* was found nearest to the isolate TV-3 (Genebank-KX538814.1) with 99% similarity. Plots treated with *T. asperellum* 2% AS at 1000 and 1200ml/ha concentration gave better disease control as the yield of green leaves as compared to Hexaconazole 5% EC. The formulation was safe to non-target beneficial organisms' in all three zones without any phytotoxicity to tea leaves at 4, 8, and 16ml/L concentrations.

**Conclusions:** The present study confirms that the developed liquid formulation of *T. asperellum* 2% AS was found significantly superior for the management of dieback disease of tea plantations in Darjeeling, Dooars, and Assam zones when used at concentrations of 1200 and 1000 ml followed by 800 and 600 ml/ha during both seasons. The maximum made tea yield was recorded in plots treated with *T. asperellum* at 1000 and 1200ml/ha followed by Hexaconazole 5% EC. The *T. asperellum* 2% AS was observed to be safe for non-target beneficial organisms viz., *C. carnea*, *O. javanus,* and *S. gilvifrons*. The developed *T. asperellum* 2% AS formulation was not phytotoxic to tea leaves at 4, 8, and 16ml/L concentrations.


# Background

Tea (*Camellia* sp) is one of the most popular non-alcoholic beverages in the world next to the water. In India, it is grown as a perennial monoculture plantation crop which may be yielding even up to 50–150 years. The young shoots comprising of two or three leaves and a bud is primarily used as the base material for manufacturing tea. Based on the fermentation process there are three varieties of tea like non-fermented (green tea), semi-fermented (oolong tea), and fermented (black tea) are consumed by people according to the preference and their taste. In India, tea is cultivated in about 6.36 lakh ha with a production of about 1,338 million kg made teas [1]. Among north-eastern states in India, Assam is well known for producing premium CTC tea and the Darjeeling area of West Bengal is famous for orthodox tea, best known as Champagne of teas and known for its aroma and flavor.

The success of tea production is limited by many biotic and abiotic factors and attack of fungal diseases like root rots, leaf spots, and stem canker is considered as one among them [2]. The dieback is a foliar disease caused by the fungi *Fusarium solani*, which infects the young shoots [3]. Management of this disease using only chemical fungicides is restricted because their excessive use may lead to unhealthy soil, pollution of groundwater, resistance development in phytopathogens, destruction of several useful organisms, and eventually cause human health problems [4, 5, 6).

Under such circumstances, the application of biopesticides becomes an alternative approach, that can take care of these diseases in tea plantations. The biopesticides are considered important as they are safer to human beings, environment, beneficial microbes besides its quickly decomposing nature [7, 8]. Among biopesticides, the fungus belonging to the genus *Trichoderma* is reported to be the best candidate managing various diseases of different crops. Among the several species of *Trichoderma*, *T. harzianum*, *H. lixii*, *T. atroviride*, *H. atroviridis*, *T. asperellum*, and *T. virens* are reported as potential biocontrol agents against phytopathogens [9, 10] and the potency of genus *Trichoderma* has been already established for the control of numerous phytopathogenic fungal genera of agricultural importance [11]. It parasitizes the phytopathogenic fungi through the detection of the host, attachment to host followed by its coiling, releasing of secondary metabolites such as antibiotics, and production of cell wall degrading enzymes [12]. *Trichoderma* produces certain compounds like isonitrile, diketopiperazines, sesquiterpenes, polyketides, alkylpyrones, and peptaibols [13]. The present study was aimed at the isolation of indigenous antagonistic fungus (*Trichoderma* sp), followed by its identification, formulation development, evaluation of its efficacy through field trials against dieback disease, besides its effect on yield, non-target organisms and phytotoxicity to tea plants for two successive years under three geographical locations *i.e.* Darjeeling, Dooars and Assam. This strain of *Trichoderma* could be commercialized after fulfilling the requirements for its registration and label claim on tea for the benefit of the tea industry for the control of dieback disease in tea plantations.

# Results

### Isolation, identification, and the accession of *Trichoderma* sp

The local fungal antagonist was isolated from the tea rhizosphere of Dooars zone, District Jalpaiguri, West Bengal, India, and was designated as KBN-29. Based on cultural and morphological characters it was identified as *T. asperellum*(Fig. 2A). Further, its identity was established as *T. asperellum* by ITCC, Division of Mycology and Plant Pathology, IARI, Pusa, New Delhi – 110012, and it has assigned an accession number as ITCC-7764. The DNA fingerprinting (16s ITS) outsourced from the National Bureau of Agriculturally Important Microorganisms, Indian Council of Agricultural Research, Kushmaur, Mau, Uttar Pradesh, India, revealed that its nucleotide sequence (Fig. 1) was the nearest similar to *T. asperellum* isolate TV-3 (Genebank-KX538814.1) possessed both query coverage and similarity 99% when followed NCBI blast. The NBAIM provided its accession number as NAIMCC-SF-0041. The liquid formulation from *T. asperellum* was prepared in the form of 2% Aqueous Suspension (AS) using a liquid fermentation technique to carry out different bioefficacy studies.

### Multilocation field Bio-efficacy of *T. asperellum* 2% AS on dieback disease during the season I and II

In the Darjeeling zone, the dieback infection(Fig. 2 B) before imposing treatment (pre-assessment) ranged from 15.78-18.11 shoots. The first spray could reduce dieback infection in all the treated plots as compared to untreated ones. Among the treatments, *T. asperellum* 2% AS at 1000 ml and 1200ml/ha concentrations were found more effective in controlling the disease with two rounds of spraying (Fig. 2 C and D) which gave superior control of disease as compared to lower concentrations (*T. asperellum* 2% AS at 600ml and 800ml/ha) and market sample (*T. harzianum* 1% WP) @2500g/ha. However, hexaconazole 5% EC exhibited the maximum disease reduction in both seasons (Table 1).

In Dooars and Assam zones, pre-assessment infestation ranged from 19.89 to 22.67 and 20.67 to 23.33, respectively in the first season. All doses of *T. asperellum* formulation yielded better over untreated control and *T. harzianum* 1% WP (market sample); however, at higher concentrations viz., 1000 ml and 1200 ml were found more effective. In the second season also a similar trend of disease control was recorded in both zones, (Table 2 and 3).

### Effect of *T. asperellum* formulation on the yield of green leaves

The average green leaf yield of first six rounds harvesting at the weekly interval in *T. asperellum* sprayed plots with 1000 ml and 1200 ml/ha was found to be significantly superior over the Hexaconazole 5% EC and *T. harzianum* 1%WP at 2500g/ha. *T. asperellum* 2% AS at 1000 ml and 1200 ml/ha showed an increased average made tea yield of 422 and 425 kg/ha, respectively during the first season and 395 and 399 kg/ha, respectively during the second season as compared to untreated control where it was 377 and 354 kg /ha in the first and second season, in Darjeeling zone. However, in Dooars zone, the yield of made tea was 1694 and 1703 Kg at concentrations of 1000 ml and 1200 ml/ha as compared to 1519 kg in control in the first year whereas it was 1724 and 1729 kg at both higher doses as compared to control (1543) in the second season. The same yield trend was observed in the Assam zone, where yield in the first year was higher (2315 and 2324 kg) at both higher concentrations as compared to control (2071 kg). In the second season, it was 2216 and 2229 kg at higher doses against 1983 kg in control during the second year (Table 4). Two higher doses of *T. asperellum* had increased the green leaf yield over control during both seasons in Darjeeling (11.93 -12.73%), Dooars (11.73 – 12.11%) and Assam (11.78 – 12.40%).

### Effect of the formulation on non-target beneficial organisms

The population level of important insect predators viz., *C. carnea*, *O. javanus,* and *S. gilvifrons* was recorded at 0 days, 7$^{th}$ day of the first spray, and 7$^{th}$, 14$^{th}$ and 21$^{st}$ day of the second spray during season 1 and 2 (Fig. 3-5). The experimental results indicated that *T. asperellum* 2% AS did not show any adverse effect on the population-level of these non-target beneficial organisms.

### Testing for phytotoxicity, tainting and organoleptic attributes

Tea leaves on the bushes were observed for phytotoxic effects after the spray of *T. asperellum* 2% AS. The test result indicated that there were no phytotoxic effects in form of wilting of leaves, vein clearing, necrosis, epinasty, and hyponasty on the tea leaves at the concentrations used in the study (Table 5).

## Discussion

In the present study, different concentrations of *T. asperellum* liquid formulation (2% AS) successfully controlled the dieback disease at all locations, and hence, it could be an alternate approach of disease management under organic crop production system of Darjeeling and other areas. Foliar spray of *Trichoderma* formulation resulted in an increased number of shoots and their length [14]. It was found that *Trichoderma* WP formulation controlled the dieback disease of tea to a great extent when sprayed at 2.5 and 5.0 g/liter concentration and performed better than the commercial formulation of the antagonist. Light pruned (LP) and deep skipped (DS) tea bushes showed enhanced vegetative growth as compared to control [15]. Foliar spray of *T. harzianum* and *T. viride* on wheat crop managed head blight disease caused by *Fusarium graminearum* under greenhouse conditions better than control [16]. *Trichoderma* spp was found to be promising under field conditions for the

management of blister blight of tea in North East India [17]. The efficacy of *T. atroviride* strains has proven for the protection of pruning wounds in the grapevine [18]. *T. asperellum* was successful in managing a broad array of fungal phytopathogens [19, 20] such as *F. oxysporum* and *Curvulariaaeria*[21, 22].

Our study showed increased production of healthy green tea shoots due to the application of *T. asperellum* liquid formulation as compared to chemical fungicide, the market sample of *T. harzianum*, and control. Hence there are chances to get bountiful production of tea crop through by applying as a foliar spray besides controlling the disease. *T. asperellum* showed synergistic activity with *Bacillus amyloliquefaciens*and combined application of both microbes significantly enhanced the growth of wheat as well as protected the crop against plant pathogens [23].

The liquid formulation of the local antagonist (*T. asperellum* 2% AS) did not adversely affect the beneficial insects in the tea ecosystem and confirmed the earlier findings that the biopesticides are less toxic, decomposes quickly, free from pollution, and residue problems. They generally affect the targeted and closely related organisms in the same environment [24, 25]. The spray of fish emulsion increased the yield of tomatoes and peppers with no observable phytotoxic effect on crop foliage under the field conditions [26]. The developed liquid formulation was found to be non-phytotoxic without showing any kind of toxicity symptoms on tea leaves.

# Methods

### Isolation, identification, and the accession of *Trichoderma* sp

Isolation of the antagonist was carried out following standard technique with slight modification [27]. Soil samples were collected from tea rhizosphere in sterilized polyethylene bags, brought to mycology laboratory, stored in the refrigerator at 4±1$^0$C. For isolation of antagonistic fungi, soil samples were homogenized processed following multiple serial dilution plate technique (MSDP). From 6$^{th}$ and 8$^{th}$ dilutions, 0.5 mL was drawn and uniformly distributed in solidified Trichoderma specific medium (HiMedia) plates in triplicates. Plates were then properly sealed with parafilm and incubated at 26±2 $^0$C for 72-96 hours. Appeared fungal colonies were observed and antagonist's colonies were identified based on its mycelia color and fast-growing character. Such colonies were transferred into another potato dextrose agar (PDA) plates for pure culture and re-incubated at 26±2 $^0$C for 96 hours in the BOD incubator.

### Identification:

The antagonistic isolate was identified based on its cultural and morphological characters by the first author. Later, its identity was re-confirmed from Indian Type Culture Collection, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, Pusa, New Delhi, India and it has assigned an accession number. To establish its perfect identity, DNA fingerprinting (16s ITS) was get done from ICAR National Bureau of Agriculturally Important Microorganisms, Indian Council of Agricultural Research, Kushmaur, Mau, Uttar Pradesh, India. The obtained nucleotide sequence blasted at the NCBI website to match the nearest similar.

### Development of formulation: *T. asperellum* 2% Aqueous Suspension

The liquid formulation of *T. asperellum* was manufactured using liquid-state fermentation techniques by M/s Varsha Bioscience and Technology India Pvt Ltd, Hyderabad - 500059, Telangana, India using the slightly modified method [28]. The mother culture of the antagonist was sub-cultured on to plates containing Trichoderma Specific Media (TSM) followed by incubation at 28±0.5°C for 5 days.

Then 10 liters of seed inocula was prepared by inoculating 120 hr old mother culture into 1000 ml conical flask filled with 250 ml autoclaved potato dextrose agar and incubated in an orbital shaker at 28±1°C and 180 rpm for seven days. From seed inocula, submerged large scale fermentation (maintained temp. 28±2 $^0$C, 45 RPM) was done to scale up its quantity using another autoclaved medium containing 30g sugarcane molasses and 5g yeast extract per liter of water. One week old seed inocula (10%) was inoculated in the fermenter incubated for 7 days and the cultural biomass was separated by centrifuging the culture broth through the on-line centrifugation system and both the conidia, as well as mycelia were collected. Active ingredient (2x10$^8$ CFU/ ml) was determined by the MSDP technique in final formulation by adding required distilled sterilized water.

### Multilocation field Bio-efficacy of *T. asperellum* 2% AS on dieback disease during the season I and II

The *T. asperellum* 2% AS formulation was tested under field conditions at Darjeeling, Dooars, and Assam zones against dieback disease caused by *F. solani* from 2016 to 2018. The plot size was kept 84 m$^2$ for each treatment with 100 bushes. Experiments were laid out in randomized block design (RBD) with seven treatments in three replications. Common tea cultivars/clones of particular zone namely TRA-AV-2, TV-25, TV-26, and TV-1 of 28 to 54 years old were chosen for the study.

The plots having dieback disease incidence above 5% ETL were selected for field bio-efficacy study. The disease incidence was recorded by placing a 1 x 1-foot quadrate at 3 randomly selected spots per treatment then healthy and infected tea shoots were plucked. Then the first spray with hand operated knapsack sprayer fitted with NMD 450 nozzle was done immediately after the plucking (0-day). Observations on disease incidence were recorded on the $7^{th}$ day of the first spray using the same quadrate. Subsequently, both healthy and infected shoots were plucked on the same day and weight (Kg) of the fresh healthy shoots was recorded. The second spray was given on the $7^{th}$ day after the $1^{st}$ spray and disease incidence was recorded on the $7^{th}$, $14^{th,}$ and $21^{st}$ days of the second spray.

**Effect of *T. asperellum* formulation on the yield of green leaves**

Green leaf yield (kg/plot) was recorded from the first six rounds of plucking and at every plucking round it was converted into made tea per hectare using the formula [29].

Made tea Kg per hectare (KMTH) = Green leaf yield (Kg) x no. of bushes/ha x Conversion Factor (0.225)

**Effect of the formulation on non-target beneficial organisms**

The population of insect predators viz., *Chrysoperlacarnea*, *Oxyopesjavanus,* and *Stethorusgilvifrons*was recorded on 0 days (pre-spray), $7^{th}$ day of $1^{st}$ spray and $7^{th}$, $14^{th}$ and $21^{st}$ day of $2^{nd}$ spray (post-spray). Visual observations were made from 30 randomly selected tea bushes per treatment to assess the population of *C. carnea* nymphs and *O. javanus* adults. Thirty leaves were collected at random per treatment and observed under a binocular microscope for assessing the population of *S. gilvifrons*.

**Testing for phytotoxicity, tainting and organoleptic attributes**

*T. asperellum* 2% AS was sprayed at a concentration of 4, 8, and 16ml per liter water to assess its phytotoxic effects on tea leaves. Three replications were maintained in 84 square meters area of the experimental plot. Observations were recorded on 0, 3, 7, and $14^{th}$ day of spray for the appearance of leaf yellowing, stunting, necrosis, epinasty, and hyponasty type symptoms and the injury level (toxic level) was rated using the following phytotoxicity rating scale (PRS) [30].

| Crop response / Crop injury (%) | Rating |
|---|---|
| 0.0 | 0 |
| 1-10 | 1 |
| 11-20 | 2 |
| 21-30 | 3 |
| 31-40 | 4 |
| 41-50 | 5 |
| 51-60 | 6 |
| 61-70 | 7 |
| 71-80 | 8 |
| 81-90 | 9 |
| 91-100 | 10 |

The percent phytotoxicity index (PPI) was computed using the following formula,

$$PPI = \frac{\text{Sum of all numerical ratings}}{\text{Number of tea plant observed x Maximum phytotoxicity rating}} \times 100$$

**Statistical analysis**

The collected data of bioefficacy trials and yield were statistically analyzed to find out the critical difference among treatment at a 5% level of significance (p =0.05) through the online statistical package “OPSTAT” of Chaudhary Charan Singh Haryana Agricultural University, Hisar (www.hau.ac.in).

## Abbreviations

AS: Aqueous Suspension; Cc: *Chrysoperla carnea;* Oj: *Oxyopes javanus;* Sg: *Stethorus gilvifrons;* ITCC: Indian Type Culture Collection; IARI: Indian Agricultural Research Institute; LP: Light pruned; DS: Deep skipped; Ta: *T. asperellum;* Th: *T. harzianum;* CC-0: *Chrysoperla carnea* on 0 day; OJ0: *Oxyopes javanus* on 0 day; SG0: *Stethorus gilvifrons* on 0 day; TRA: Tea Research Association; NBAIM: National Bureau of Agriculturally Important Microorganisms

## Declarations

### Ethics approval and consent to participate

The study was conducted using different tea plant species those are abundant in the ecosystem hence do not require ethical approval.

### Consent for publication

The authors agree to publish this paper. The data has not been published partially or completely in any other journal.

### Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to privacy reasons but are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper. It is declared that the authors have no competing interests.

### Funding

At present we do not have any financial support from any funding agency for this research work to get it published. However, the waivers (BMC Plant Biology Journal) considered it for the review process without processing charges.

### Authors’ contributions

KCK: Isolated, identified the *T. asperellum*, developed SOP for field trials, tabulated results, and written the manuscript. AB: Decided the experimental locations, analyzed the data, and provided overall guidance. JPA: Developed protocol for product formulation of antagonist and made it available for field trials. BD: Shaped the manuscript as per the Journal’s format. MB: Conducted field trials in selected zones. HR and PD: Collected data of all experiments. All authors read and approved the final manuscript.

### Acknowledgments

The authors are highly thankful to the Biotechnology Industry Research Assistance Council (BIRAC) for sanctioning the research project under the CRS scheme to develop local strains of antagonist and entomopathogenic fungi. We are thankful to Dr. Prameela Devi and Dr. Deeba Kamil, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI) Pusa, New Delhi, for their help in the identification of *Trichoderma* strain and assigning an accession number as ITCC-7764. The DNA fingerprinting done through outsourcing with the accession number NAIMCC-SF-0041from ICAR-NBAIM, Kushmaur, Mau, Uttar Pradesh- 275103, India, is also acknowledged.

## References

1. Tea Board India. 2018. http://www.teaboard.gov.in/pdf/Area_1_pdf2863.pdf /Production_Region_wise_pdf2736.pdf. Accessed 4 August 2020.
2. Kumhar KC, Babu A. Economically important diseases of tea (*Camellia* sp) and their management strategies. In: Chand G, Akhtar MN, Kumar S, editors. Diseases of fruits and vegetable crops: Recent management approaches. Apple Academic Press & CRC Press Taylor & Francis Group; 2020.p. 435-459.

3. Barthakur BK, Dutta P. 2011. Disease management in tea. In: Goswami BK, editor. Tea field management. Tea Research Association, Tocklai Experimental Station, Jorhat, Assam: 2011.p. 182- 188.
4. Sarwar M. The killer chemicals as controller of agriculture insect pests: the conventional insecticides. Int J Chem Biomol Sci. 2015; 1: 141–147.
5. Garcia-Garcia CR, Parron T, Requena M, Alarcon R, Tsatsakis AM, Hernandez AF. Occupational pesticide exposure and adverse health effects at the clinical, hematological and biochemical level. Life Sci. 2016; 145: 274–283. https://doi.org/10.1016/j.lfs.2015.10.013.
6. Shammi M, Sultana A, Hasan N, Mostafizur Rahman M, Saiful Islam M, Bodrud-Doza M, Khabir Uddin M. Pesticide exposures towards health and environmental hazard in Bangladesh: a case study on farmers' perception. J Saudi Soc Agric Sci. 2018. https://doi.org/10.1016/j.jssas.2018.08.005.
7. Leahy J, Mendelsohn M, Kough J, Jones R, Berckes N. 2014. Biopesticide oversight and registration at the U.S. Environmental Protection Agency, In: ACS Symposium Series. pp. 3–18. DOI: 10.1021/bk-2014-1172.ch001.
8. Villaverde JJ, Sevilla-Moran B, Sandin-Espana P, Lopez-Goti C, Alonso-Prados JL. Biopesticides in the framework of the European Pesticide Regulation (EC) No.1107/2009. Pest Manage Sci. 2014; 70: 2–5. https://doi.org/10.1002/ps.3663.
9. Jeger MJ, Jeffries P, Elad Y, Xu XM. A generic theoretical model for biological control of foliar plant diseases. J Theor Biol. 2009; 256:201-214.
10. Hjeljord L, Tronsmo A.1998. *Trichoderma* and *Gliocladium* in biological control: an overview. *Trichoderma* and *Gliocladium*, Enzymes, Biological Control and Commercial Applications, Edited by Kubicek CP, Harman GE, Taylor and Francis, London, UK. 2:131- 151.
11. Contreras-Cornejo H A, Macias-Rodriguez L, Del-Val E, Larsen J. Ecological functions of *Trichoderma* and their secondary metabolites in the rhizosphere: interactions with plants. FEMS Microbiol Ecol. 2016; 92:fiw036. doi: 10.1093/femsec/fiw036.
12. Zeilinger S, Gruber S, Bansal R, Mukherjee PK. Secondary metabolism in *Trichoderma* – chemistry meets genomics. Fungal Biol Rev. 2016; 30: 74–90.
13. Patil AS, Patil SR, Paikrao HM. 2016. "*Trichoderma* Secondary Metabolites: Their Biochemistry and Possible Role in Disease Management," in Microbial-Mediated Induced Systemic Resistance in Plants, eds D. K. Choudhary and A. Varma (Singapore: Springer Singapore): 69–102.
14. Kumhar KC, Babu A. *In vitro* study on bio-efficacy, fungicide tolerance and shelf life of local isolate of *Trichoderma viride.* Two and a Bud. 2015; 62(2): 17-20.
15. Kumhar KC, Babu A, Arulmarianathan JP, Deka B, Bordoloi M, Rajbongshi HJ, Dey P. Role of beneficial fungi in managing diseases and insect pests of tea plantation. Egyptian Journal of Biological Pest Control. 2020; 30(78): 1-9.
16. Panwar V, Aggarwal A, Singh G, Verma A, Sharma I, Saharan MS. Efficacy of foliar spray of *Trichoderma* isolates against *Fusarium graminearum*causing head blight of wheat. J Wheat Res. 2014; 6(1): 59-63.
17. Sarmah SR, Dutta P, Begum R, Tanti AJ, Phukan I, Debnath S, Barthakur BK. 2005. International Symposium on Innovation in Tea Science and Sustainable Development in Tea Industry. Proc. China Tea Science.
18. Mondello V, Larignon P, Armengol J, Kortekamp A, Vaczy K, Prezman F, Serrano E, Rego C, Mugnai L, Fontaine F. Management of grapevine trunk diseases: knowledge transfer, current strategies and innovative strategies adopted in Europe. PhytopatholMediterr. 2018;57: 369–383. https://doi.org/10.14601/Phytopathol_ Mediterr-23942.
19. Marcello CM, Steindorff AS, Da Silva SP, Silva Rdo N, Mendes Bataus LA, Ulhoa CJ. Expression analysis of the exo-beta-1,3-glucanase from the mycoparasitic fungus *Trichoderma asperellum*. Microbiol Res. 2010; 165: 75–81. doi: 10.1016/j.micres.2008.08.002.
20. Wu Q, Zhang L, Xia H, Yu C, Dou K, Li Y. Omics for understanding synergistic action of validamycin and *Trichoderma asperellum* GDFS1009 against maize sheath blight pathogen. Sci Rep. 2017; 7:40140. doi: 10. 1038/srep40140.
21. Baiyee B, Pornsuriya C, Ito SI, Sunpapao A. *Trichodermaspirale* T76-1 displays biocontrol activity against leaf spot on lettuce (Lactuca sativa L.) caused by *Corynesporacassiicola* or *Curvulariaaeria*. Biol Control. 2019; 129: 195–200.
22. Veenstra A, Rafudeen MS, Murray SL. *Trichodermaasperellum* isolated from African maize seed directly inhibits *Fusariumverticillioides* growth in vitro. Eur J Plant Pathol. 2019; 153: 279–283.
23. Karuppiah V, Sun J, Li T, Vallikkannu M, Chen J. Co-cultivation of *Trichodermaasperellum* GDFS1009 and *Bacillusamyloliquefaciens* 1841 causes differential gene expression and improvement in the Wheat growth and biocontrol activity. Front Microbiol. 2019; 10:1068. doi: 10.3389/fmicb.2019.01068.
24. Leahy J, Mendelsohn M, Kough J, Jones R, Berckes N. 2014. Biopesticide oversight and registration at the U.S. Environmental Protection Agency, In: ACS Symposium Series. pp. 3–18. DOI: 10.1021/bk-2014-1172.ch001.
25. Villaverde JJ, Sevilla-Morán B, Sandín-España P, López-Goti C, Alonso-Prados JL. Biopesticides in the framework of the European Pesticide Regulation (EC) No. 1107/2009. Pest Manage Sci. 2014; 70: 2–5. https://doi.org/10.1002/ps.3663.

26. Abbasi PA, Cuppels DA, Lazarovits G. Effect of foliar applications of neem oil and fish emulsion on bacterial spot and yield of tomatoes and peppers. Can J Plant Pathol. 2003; 25: 41–48.
27. Askew DJ, Laing MD. An adapted selective medium for the quantitative isolation of *Trichoderma* Plant Pathol. 1993; 42: 686-90.
28. Khan S, Bagwan NB, Iqbal MA, Tamboli RR. Mass multiplication and shelf life of liquid fermented final product of *Trichoderma viride* in different formulations. Advances in Bioresearch. 2011; 2(1): 178 – 182.
29. Ponmurugan P, Baby UI. Evaluation of fungicides and biocontrol agents against Phomopsis canker of tea under field conditions. Australasian Plant Pathol. 2007; 36: 68-72.
30. Rajeswaran J, Santharam G, Chandrasekran S. Studies on compatibility and phytotoxocity of carbosulfan 25 EC with certain agrochemicals on cotton. J Ent Res. 2004; 28(3):247-252.

# Tables

Table 1
Bio-efficacy of *T. asperellum* 2% AS against dieback disease under field conditions in Darjeeling Zone

| Treatment | Dose (ml or g / ha) | Dieback incidence* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Season I | | | | | Season II | | | | |
| | | No of infected shoots | | | | | | | | | |
| | | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray |
| T1-*Ta* 2% AS | 600 | 15.89 | 13.56 $^{cd}$ | 13.00$^{b}$ | 12.89$^{c}$ | 11.78$^{c}$ | 19.78 | 15.89 $^{cd}$ | 14.44$^{b}$ | 13.11 $^{cd}$ | 11.89$^{c}$ |
| T2-*Ta* 2% AS | 800 | 16.44 | 13.11$^{bc}$ | 12.33$^{b}$ | 11.11$^{b}$ | 10.33$^{b}$ | 20.67 | 14.89$^{bc}$ | 13.67$^{b}$ | 11.56$^{bc}$ | 10.44$^{b}$ |
| T3-*Ta* 2% AS | 1000 | 15.78 | 11.78$^{ab}$ | 10.66$^{a}$ | 8.00$^{a}$ | 7.89$^{a}$ | 19.44 | 13.44$^{ab}$ | 11.22$^{a}$ | 8.56$^{a}$ | 8.11$^{a}$ |
| T4-*Ta* 2% AS | 1200 | 15.89 | 11.33$^{a}$ | 10.33$^{a}$ | 7.78$^{a}$ | 7.56$^{a}$ | 20.56 | 12.89$^{a}$ | 10.89$^{a}$ | 8.22$^{a}$ | 7.33$^{a}$ |
| T5-*Th* 1% WP** | 2500 | 17.22 | 15.00$^{d}$ | 14.56$^{c}$ | 14.33$^{c}$ | 13.56$^{d}$ | 20.33 | 16.89$^{d}$ | 15.67$^{c}$ | 14.67$^{d}$ | 13.78$^{d}$ |
| T6-Hexaconazole | 400 | 17.56 | 11.00$^{a}$ | 10.00$^{a}$ | 9.89$^{b}$ | 9.78$^{b}$ | 20.89 | 12.56$^{a}$ | 10.44$^{a}$ | 10.22$^{ab}$ | 9.89$^{b}$ |
| T7-Untreated control | - | 18.11 | 20.11$^{e}$ | 22.56$^{d}$ | 25.00$^{d}$ | 27.33$^{e}$ | 22.00 | 24.22$^{e}$ | 25.78$^{d}$ | 27.89$^{e}$ | 29.89$^{e}$ |
| **C.D** | **-** | **2.41** | **1.71** | **1.34** | **1.54** | **1.30** | **2.67** | **1.74** | **1.06** | **2.12** | **1.31** |

***Values represent mean of three replications, **Market sample**

Table 2
Bio-efficacy of *T. asperellum* 2% AS against dieback disease under field conditions in Dooars Zone

| Treatment | Dose (ml or g / ha) | Dieback incidence* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Season I | | | | | Season II | | | | |
| | | No of infected shoots | | | | | | | | | |
| | | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray |
| T1-*Ta* 2% AS | 600 | 21.44 | 15.78 $^{cd}$ | 15.11$^{c}$ | 14.22$^{c}$ | 13.11$^{d}$ | 17.78 | 14.11$^{c}$ | 14.00$^{c}$ | 12.33$^{c}$ | 11.67$^{c}$ |
| T2-*Ta* 2% AS | 800 | 19.89 | 14.89$^{bc}$ | 13.44$^{b}$ | 11.89$^{b}$ | 10.78$^{c}$ | 19.11 | 13.44$^{bc}$ | 12.56$^{b}$ | 10.44$^{b}$ | 9.33$^{b}$ |
| T3-*Ta* 2% AS | 1000 | 20.00 | 14.00$^{ab}$ | 12.56$^{a}$ | 9.89$^{a}$ | 8.56$^{a}$ | 18.44 | 12.45$^{ab}$ | 11.11$^{a}$ | 8.45$^{a}$ | 7.78$^{a}$ |
| T4-*Ta* 2% AS | 1200 | 20.78 | 13.56$^{a}$ | 12.33$^{a}$ | 9.78$^{a}$ | 8.22$^{a}$ | 18.67 | 12.00$^{a}$ | 10.56$^{a}$ | 8.22$^{a}$ | 7.22$^{a}$ |
| T5-*Th* 1% WP** | 2500 | 20.89 | 16.44$^{d}$ | 15.67$^{c}$ | 14.77 $^{c}$ | 13.56$^{d}$ | 19.33 | 15.78$^{d}$ | 15.67$^{d}$ | 14.22$^{d}$ | 13.44$^{d}$ |
| T6-Hexaconazole | 400 | 20.67 | 13.11$^{a}$ | 12.00$^{a}$ | 10.78$^{ab}$ | 9.44$^{b}$ | 18.89 | 11.67$^{a}$ | 10.22$^{a}$ | 9.00$^{ab}$ | 8.89$^{b}$ |
| T7-Untreated control | - | 22.67 | 24.67$^{e}$ | 26.56$^{d}$ | 28.89$^{d}$ | 31.33$^{e}$ | 20.78 | 23.11$^{e}$ | 25.56$^{e}$ | 27.56$^{e}$ | 29.89$^{e}$ |
| **C.D** | **-** | **2.82** | **0.97** | **0.81** | **1.11** | | **2.39** | **1.30** | **1.40** | **1.59** | **1.01** |

***Values represent mean of three replications, **Market sample**

Table 3
Bio-efficacy of *T. asperellum* 2% AS against dieback disease under field conditions in Assam Zone

| Treatment | Dose (ml or g / ha) | Dieback incidence* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Season I | | | | | Season II | | | | |
| | | No of infected shoots | | | | | | | | | |
| | | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray | Pre-spray | 7th day after 1st spray | 7th day after 2nd spray | 14th day after 2nd spray | 21st day after 2nd spray |
| T1-*Ta* 2% AS | 600 | 21.78 | 16.78$^{c}$ | 15.89$^{bc}$ | 15.00$^{d}$ | 14.00$^{d}$ | 23.67 | 18.22 $^{cd}$ | 17.11 $^{cd}$ | 14.67$^{c}$ | 12.33$^{d}$ |
| T2-*Ta* 2% AS | 800 | 22.00 | 16.00$^{bc}$ | 15.11$^{b}$ | 13.22$^{c}$ | 11.89$^{c}$ | 24.44 | 16.89$^{bc}$ | 15.78$^{bc}$ | 12.56$^{b}$ | 10.78$^{c}$ |
| T3-*Ta* 2% AS | 1000 | 20.67 | 14.56$^{ab}$ | 13.33$^{a}$ | 9.78$^{a}$ | 8.67$^{a}$ | 23.00 | 15.33$^{ab}$ | 13.67$^{a}$ | 10.33$^{a}$ | 8.56$^{a}$ |
| T4-*Ta* 2% AS | 1200 | 21.44 | 14.00$^{a}$ | 13.00$^{a}$ | 9.56$^{a}$ | 8.33$^{a}$ | 22.89 | 14.89$^{a}$ | 12.89$^{a}$ | 9.89$^{a}$ | 8.00$^{a}$ |
| T5-*Th* 1% WP** | 2500 | 21.22 | 17.56$^{c}$ | 16.89$^{c}$ | 16.33$^{e}$ | 14.67$^{d}$ | 24.22 | 19.00$^{d}$ | 18.00$^{d}$ | 16.33$^{d}$ | 13.56$^{d}$ |
| T6-Hexaconazole | 400 | 21.89 | 13.78$^{a}$ | 12.67$^{a}$ | 11.34$^{b}$ | 10.00$^{b}$ | 23.78 | 14.22$^{a}$ | 12.78$^{a}$ | 11.22$^{ab}$ | 9.67$^{b}$ |
| T7-Untreated control | - | 23.33 | 25.33$^{d}$ | 26.89$^{d}$ | 29.22$^{f}$ | 30.44$^{e}$ | 25.67 | 28.33$^{e}$ | 29.89$^{e}$ | 31.78$^{e}$ | 33.66$^{e}$ |
| **C.D** | **-** | **2.79** | **1.68** | **1.66** | **1.16** | **1.10** | **3.02** | **1.61** | **1.52** | **1.48** | **1.02** |

***Values represent mean of three replications, **Market sample**

Table 4
Effect of *T. asperellum* 2%AS on tea yield

| Treatment details | Dose/ha | Darjeeling | | | | Dooars | | | | Assam | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Season-1 (2016) | | Season-2 (2018) | | Season-1 (2016) | | Season-2 (2018) | | Season-1 (2017) | | Season-2 (2018) | |
| | | A* | B | A* | B | A* | B | A* | B | A* | B | A* | B |
| T1-*Ta* 2% AS | 600 ml | 2.60$^{c}$ | 403 | 2.43$^{c}$ | 377 | 10.64 cd | 1652 | 10.68c | 1658 | 14.39d | 2234 | 13.79 cd | 2141 |
| T2-*Ta* 2% AS | 800 ml | 2.63$^{bc}$ | 408 | 2.46$^{c}$ | 382 | 10.71bc | 1663 | 10.89b | 1691 | 14.54c | 2258 | 13.93bc | 2163 |
| T3-*Ta* 2% AS | 1000 ml | 2.72$^{a}$ | 422 | 2.55$^{a}$ | 395 | 10.91a | 1694 | 11.10a | 1724 | 14.91a | 2315 | 14.28a | 2216 |
| T4-*Ta* 2% AS | 1200 ml | 2.74$^{a}$ | 425 | 2.57$^{a}$ | 399 | 10.97a | 1703 | 11.14a | 1729 | 14.97a | 2324 | 14.36a | 2229 |
| T5-*Th* 1% WP** | 2500 g | 2.55$^{d}$ | 396 | 2.39$^{d}$ | 371 | 10.55d | 1638 | 10.53d | 1635 | 14.25e | 2212 | 13.72d | 2131 |
| T6-Hexaconazole | 400 ml | 2.67$^{b}$ | 415 | 2.51$^{b}$ | 390 | 10.79b | 1675 | 10.98b | 1705 | 14.79b | 2296 | 14.08b | 2185 |
| T7-Untreated control | - | 2.43$^{e}$ | 377 | 2.28$^{e}$ | 354 | 9.78e | 1519 | 9.94e | 1543 | 13.34f | 2071 | 12.77e | 1983 |
| **C.D** | - | **0.04** | **-** | **0.03** | **-** | **0.10** | - | **0.09** | - | **0.10** | - | **0.19** | - |

***A - Green leaf yield (Kg/Plot) obtained from six plucking, B- Made tea yield (kg/ha/year), ** Market sample**

**Table 5: Evaluation of phytotoxicity of *T. asperellum* 2% AS on tea plants at Darjeeling, Dooars and Assam zone**

| Treatments | Phytotoxicity observations during 1st and 2nd year | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Leaf tip | | Leaf surface | | Wilting of leaf | | Vein clearing | | Necrosis | | Epinasty | | Hyponasty | |
| | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year |
| | Observations before the treatment spray (day-0) | | | | | | | | | | | | | |
| T1- *Ta* 2% AS 4 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T2- *Ta* 2% AS 8 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T3- *Ta* 2% AS 16 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Observations 3 days after treatment spray | | | | | | | | | | | | | |
| T1- *Ta* 2% AS 4 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T2- *Ta* 2% AS 8 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T3- *Ta* 2% AS 16 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Observations 7 days after treatment spray | | | | | | | | | | | | | |
| T1- *Ta* 2% AS 4 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T2- *Ta* 2% AS 8 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T3- *Ta* 2% AS 16 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Observations 14 days after treatment spray | | | | | | | | | | | | | |
| T1- *Ta* 2% AS 4 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T2- *Ta* 2% AS 8 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T3- *Ta* 2% AS 16 ml/L | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

## Figures

TCACATCAGAAAGTTGGGTGTTTTACGGACGTGGACGCGCCGCGCTCCCGGTGCGAGTTT
GCAAACTACTCCAGGAGAGGCTGCGGCGAGACCGCCACTTATTICGGGGCCGGCACCCTG
TGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGAGGGGTTCGAGGGTTGAAATGACGCT
CGGACAGGCATGCCCGCCAGAATACTGGCGGGCGCAATTGCGTTCAAAGATTOGATGATT
CACTGAATICTCAATTCACATTACTTATCGCATCCTCGTICTTCATCGATGCCAGAACCAAG
AGATCCGTIGTTGAAAGTTTTGATTCATTTIGAATTITTGCTCAGAGCTGTAAGAAATACGTC
CGCGAGGGGACTACAGAAAGAGTTTGCTTGGTTCCTCGGCGGGCGCCTGGTTCCGGGGCT
GCGACGCACCCGGGGCTGACCCCGCCGAGGCAACAGTTTGGTAACGTTCACATTGGTTTG
GGAGTTGTAAACTCGGTAATGATCCCTCCGCAGGTTCACCTACGGAA

Figure 1


**Figure 2. Performance of *T. asperellum* strain on tea plantation**
**A- *T. asperellum* strain, B- Diseased shoot, C- Sprayed bushes, D-Post spray emergence of healthy shoots**

Figure 2

Figure 2

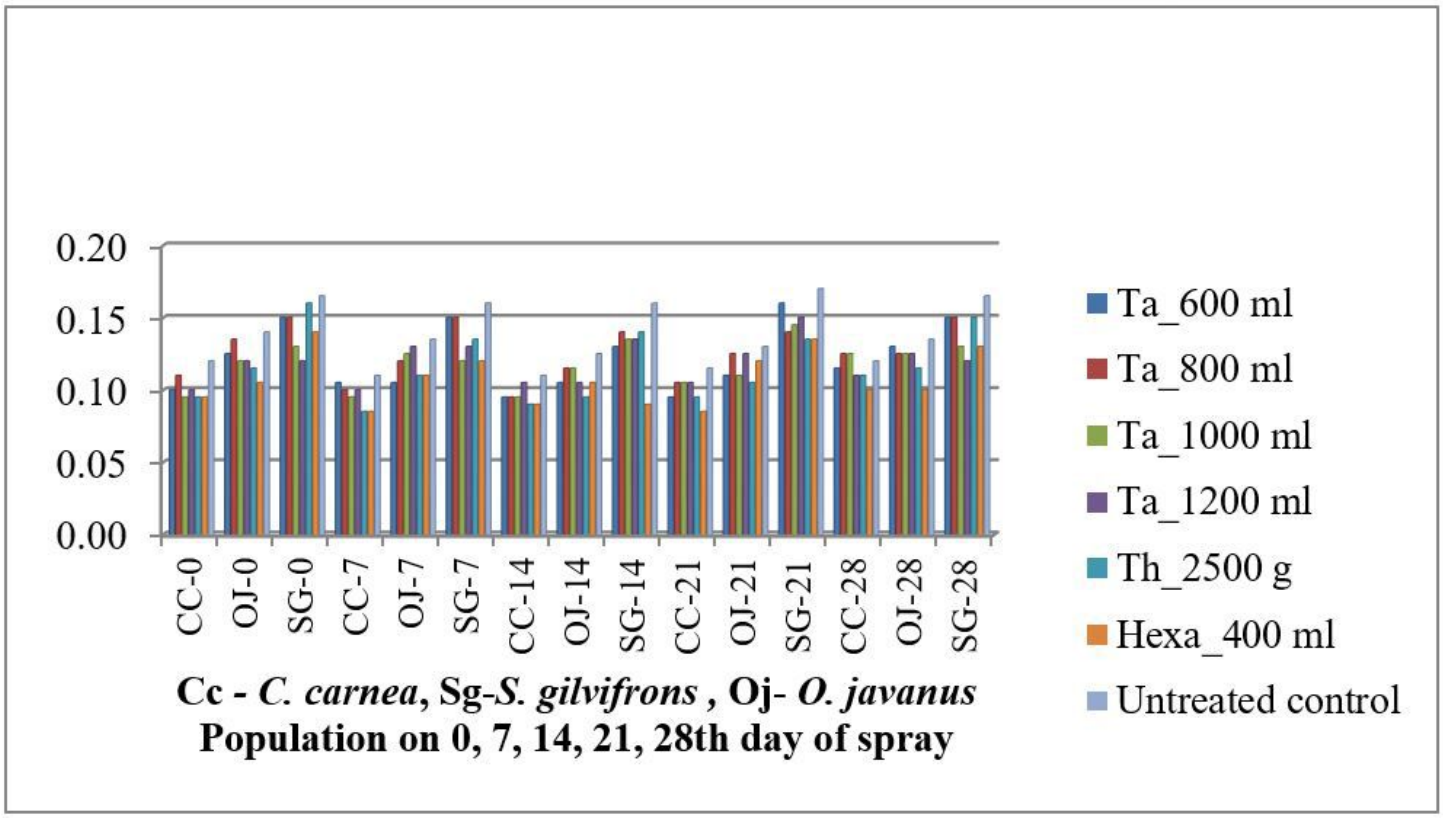


Figure 3

Effect of T. asperellum 2% AS on non-target beneficial organisms in tea in Darjeeling (Average of 2 years )

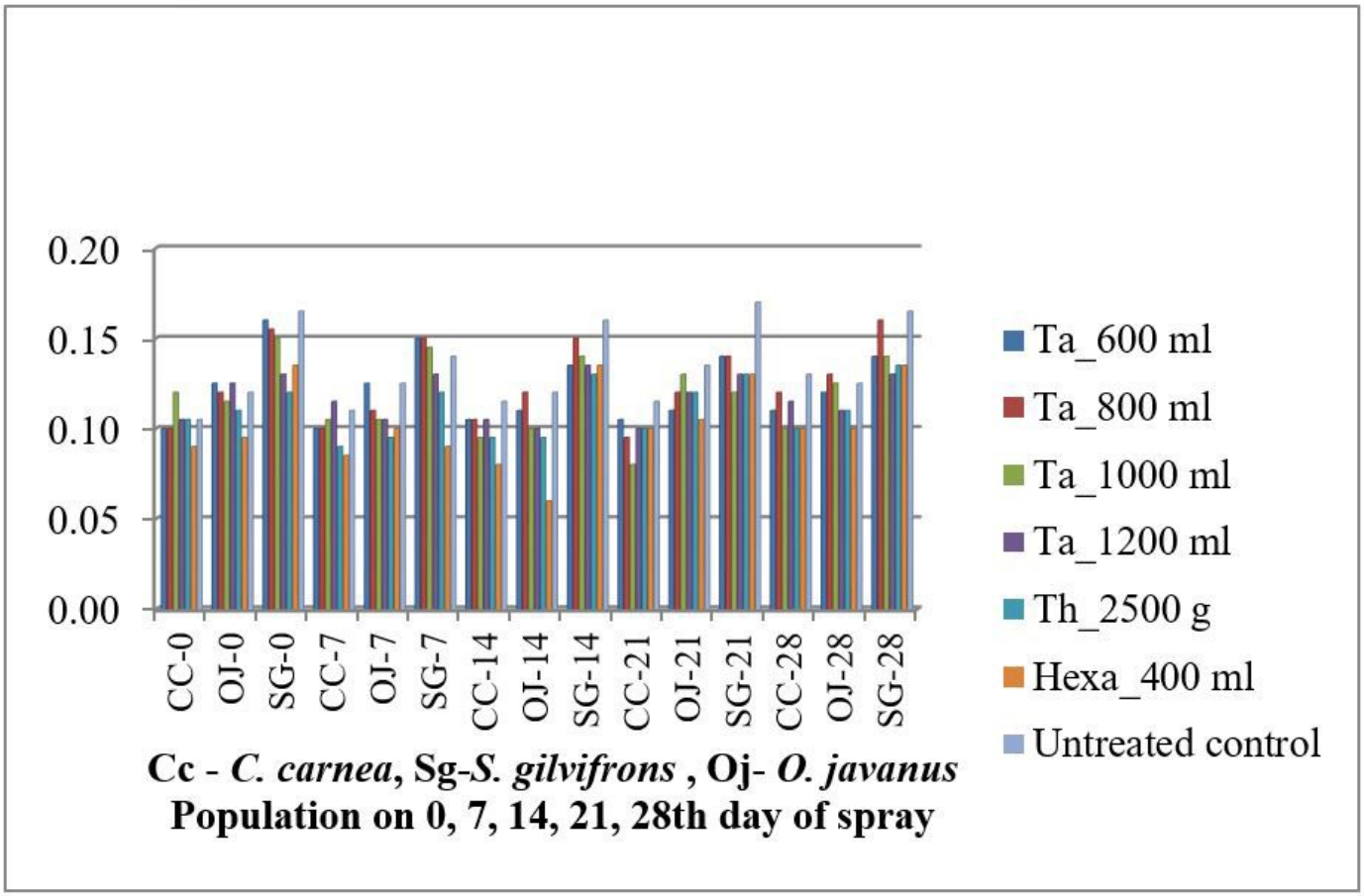


Figure 4

Effect of T . asperellum 2% AS on non-target beneficial organisms in tea in Dooars (Average of 2 years)

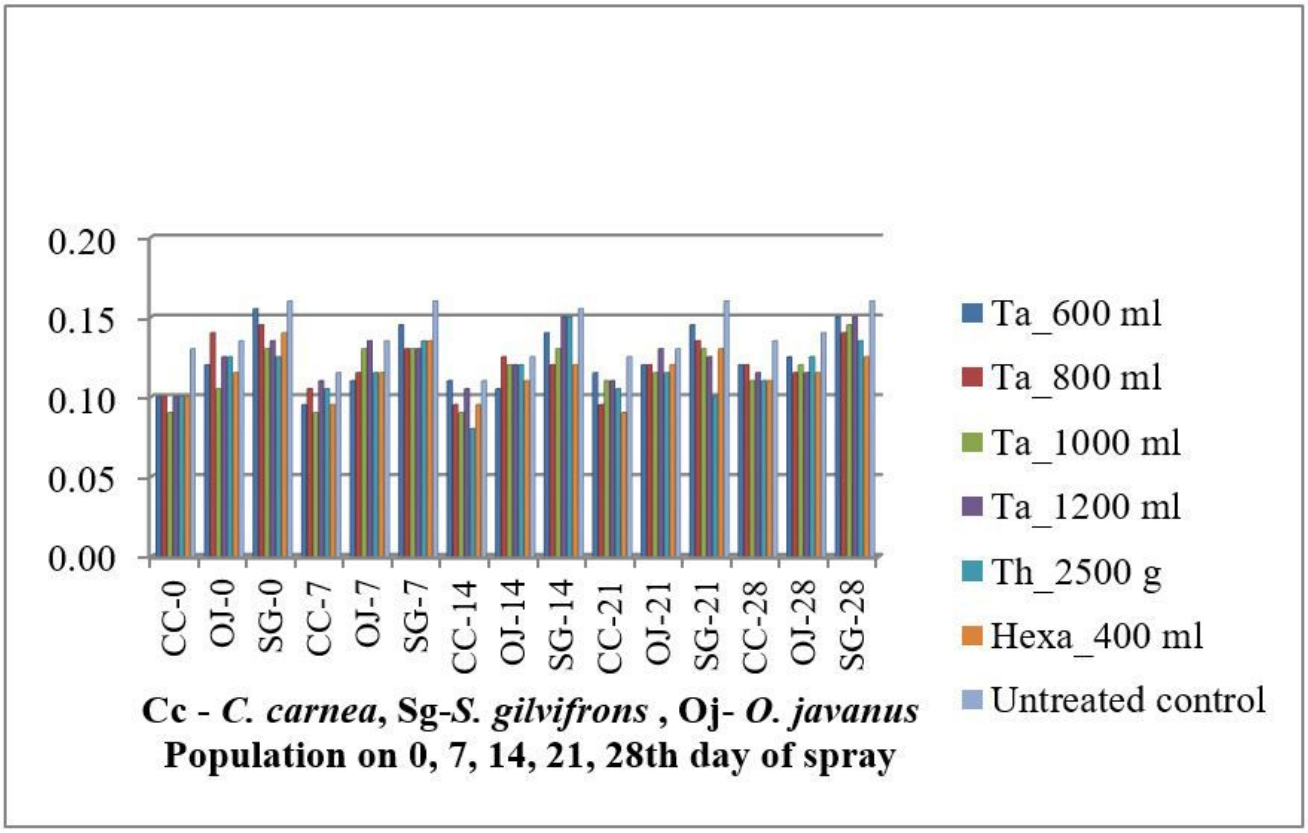


Figure 5

Effect of T . asperellum 2% AS on non-target beneficial organisms in tea in Assam (Average of 2 years)