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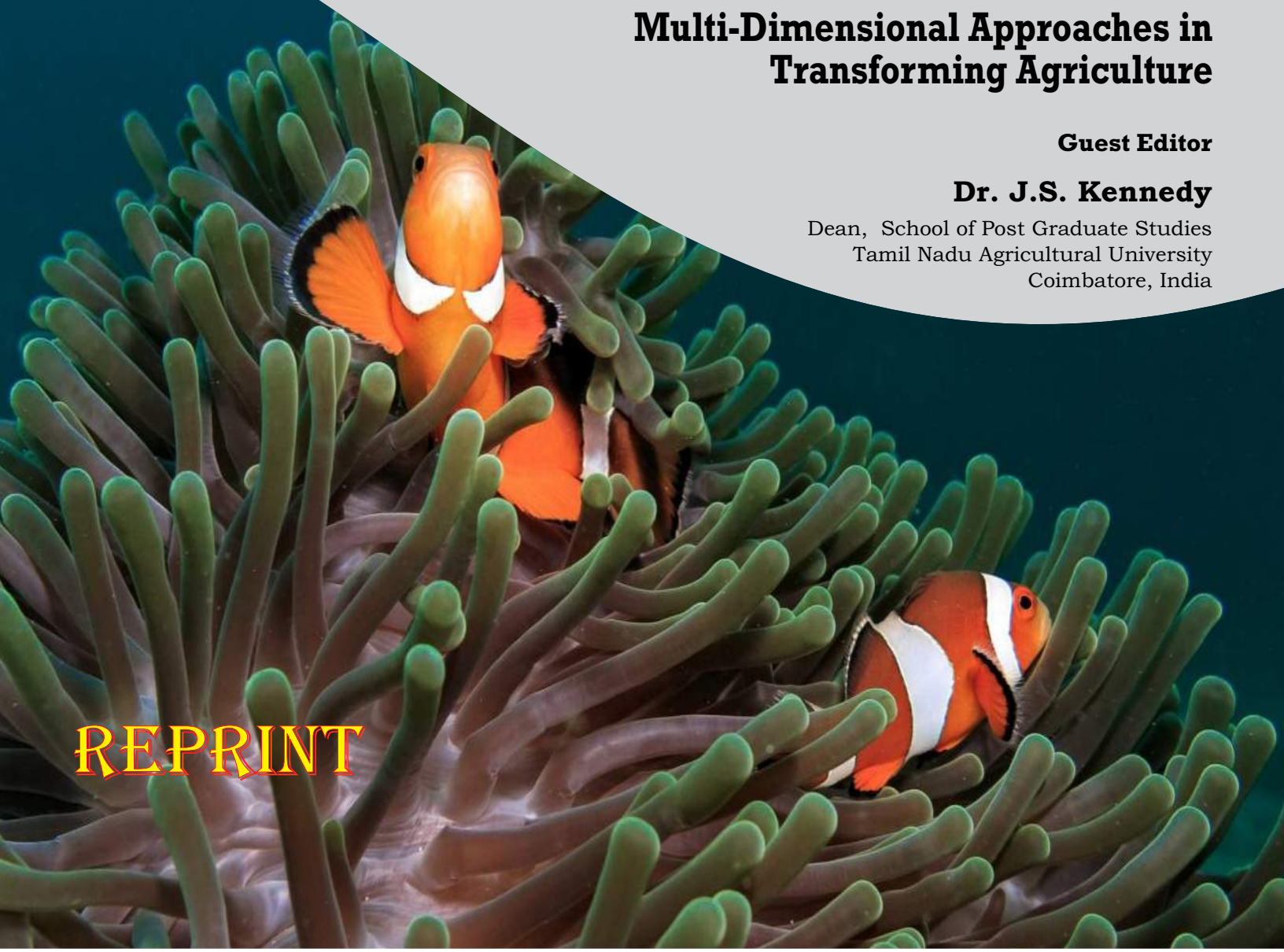
Multi-Dimensional Approaches in Transforming Agriculture

Guest Editor

Dr. J.S. Kennedy

Dean, School of Post Graduate Studies
Tamil Nadu Agricultural University
Coimbatore, India



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Guest Editor

Dr. J.S. Kennedy holds Doctor en Science degree from Université Catholique de Louvain, Louvain-la-Neuve, Belgium and Post Doc from University of Rochester, New York, USA. He worked in several Scientific Capacities at Tamil Nadu Agricultural University, Coimbatore. Currently, he is serving as Dean, School of Post Graduate Studies, Tamil Nadu Agricultural University, Coimbatore. Dr. J.S. Kennedy has about one hundred and twenty publications in international journals to his credit. His research interests are broad, including Population Ecology, Biological Control, Insect Pathology, Climatology and Insect pest Management. His researches on Host-Parasitoid Coevolution and Endosymbiont mediated defences are noteworthy. He has collaborated with several researchers Nationally and Internationally. His contribution includes Development and Quality Control of Microbial Pesticides, Establishment of Insect Tissue Culture, Impact of Temperature and Carbon dioxide on Insect Population, Endophytes for Pest Management, Mapping Bruchid resistance and Endosymbiont mediated defences. Moreover, Dr. J.S. Kennedy has several scholastic honours and professional distinctions to his credit that includes Young Scientist 1999 by Applied Zoologist Association. With all these achievements, the younger generation will surely look up to Dr. J.S. Kennedy as a role model.

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The Editorial Board of *Journal of Environmental Biology* comprises highly qualified academic leaders from institutions worldwide. The members of Editorial Board assist in managing the peer review & editing processes of submitted manuscripts.

It is hoped that the journal will achieve greater scientific excellence with their cooperation and expertise.

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Research & Development Division

The Research and Development (R&D) Division is a unique feature of the organization. a team of expert scientists in this division keeps pace with the latest advancements made in different research fields, scrutinise reviewed and edited papers for unethical practices and technical quality at each stage of publication to enhance the scientific merit of the research papers.

Panel of Editors & Reviewers of 42 (4 Supplement) July 2021

The following scientists acted as reviewers for the papers published in July issue of the Journal i.e., **42 (4 Supplement) 2021**. *Journal of Environmental Biology* thank all of the accomplished scientists in listed underneath for their valued services.

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Multi-Dimensional Approaches in Transforming Agriculture

Dr. J. S. Kennedy

Dean, School of Post Graduate Studies, Tamil Nadu Agricultural University, Coimbatore – 641 003, India

Navigating the complexity of a transformation is invariably tough for Governments, even though they may prioritize agricultural investment and recognize how important it is to get right. This is especially true in an era where Governments are seeking Agricultural Transformations that meet multiple goals simultaneously. In addition to traditional economic development and poverty reduction goals, Governments are also focusing on Agricultural Transformation plans based on Sustainable Development Goals (SDGs) by considering, for example, climate-smart strategies, women's economic empowerment and biodiversity. The drivers of Agricultural Transformation are multidimensional, interrelated and have changed over time. Still, they can be organized into categories to provide better opportunities for pragmatic diagnostics and decision making on national priorities.

Indian agriculture always provides lot of interwoven challenges to the growing farming community. Farmers today face a complicated host of environmental, social and economic pressures: protecting water and air resources, mitigating greenhouse gases, conserving biodiversity and limiting soil erosion, all while trying to make a living. These challenges are linked, yet most agricultural research of the last 80 years has approached them from a reductionist standpoint. To build truly sustainable farming systems, agricultural research must embrace through multidimensional approaches. Multidimensional approaches for Agriculture outlines both the theory and practice of agricultural systems research, helping agricultural professionals to study, understand and develop economically, socially and environmentally sustainable production systems. There is need to enhance the crop productivity through the changing innovative breeding and crop management strategies. Environmental impacts on farm food security are largely determined by various factors. Agricultural natural resources include air, minerals, plants, soil and water. Conservation is the care and protection of these resources so that they can persist for future generations. It includes maintaining diversity of species, genes, and agro ecosystems, as well as functions of the environment, such as nutrient cycling. Conservation Agriculture is similar to preservation, but while both relate to the protection of nature, they strive to accomplish this task in different ways. Conservation Agriculture seeks the sustainable farm use of nature by humans, for activities while preservation means protecting nature. Current research efforts include agricultural water quality and management, engineering for economically and environmentally sound animal production systems, grain handling and food processing, agricultural machine design and automated controls, precision farming systems, agricultural safety, seed conditioning and processing, and soil tillage and management systems. Research is being directed increasingly towards bio systems engineering through the use of biosensors, image analysis, biological systems modeling, and the design and control of biological systems and processes. Bio renewable and biofuel products and processes are an important focus of these research efforts.

The social dimension programmes for food security and nutrition should be guided by human rights norms and standards and should be complemented as appropriate by policies, guidelines and legislation. In this context, nutraceuticals play an important standpoint for improving the rural health. Traditionally, many health based preparations are being consumed by Indian consumers regularly. But due to current emerging situation of infectious diseases there is a need to look for more nutraceutical based diet to strength the immunity in India.

Keeping in view of all these facts, the Tamil Nadu Agricultural University (TNAU), Coimbatore organized the 6th Agricultural Graduate Students' Conference (AGSC) 2020 during May 28-29, 2020 by online mode for the first time on "Multi-Dimensional Approaches in Transforming Agriculture", with a key focus essentially on eight interdisciplinary themes such as Crop Productivity Enhancement: Integrating Breeding and Crop Management, Environmental Impacts on Food Security, Preserving and Protecting Natural

Resources, Social Dimensions in Improving Crop Productivity, Innovations in Agricultural and Biosystem Engineering, Approaches in Agricultural Biotechnology and Nanotechnology, Protective Cultivation and Vertical Farming and Nutraceuticals in Rural Health Improvement to offer solutions for transforming Agriculture.

COVID-19 pandemic did not deter the students and faculties to organize this mega event in which 258 extended abstracts were accepted for oral and poster presentations under 8 theme areas and also published as Conference Proceedings with ISBN. In total, 253 participants registered for the online session. This conference served as a unique platform and offered a stimulating venue for student research exchange and provided opportunity to broaden their social and scientific network. Considering its importance, Dr. N. Kumar, Vice-Chancellor, TNAU Coimbatore presided over the inaugural session remotely from Chennai and Dr. R.C. Agrawal, DDG (Education), ICAR provided his special keynote address. Seventeen invited speakers remotely participated from different parts of the world like USA, Italy, Canada, Australia and West Indies.

The technical committee comprising of subject experts decided to publish the research articles in a journal with NAAS score of 6.0 or above so as to encourage the students who have undertaken outstanding research work. Consequently, a proposal was sent to the Editor-in-chief of *Journal of Environmental Biology* (JEB), with NAAS score >6.0 and also indexed in various databases expressing interest to publish the articles in JEB as Special/Supplement Issue.

The R&D Division of *J. Environ. Biol.* short-listed 20 research papers. These papers were sent for peer review. On the recommendation reports of the reviewers and further clearance from the R&D division, 14 papers finally were accepted for publication in *Journal of Environmental Biology.*

I as a Guest Editor of this Special/Supplement Issue, profusely wish to thank and appreciate our team members Dr. N.O. Gopal, Dr. M. Raveendran, Er. G. Vanitha, Dr. S.K. Rajkishore and Dr. R. Prabhu for their meticulous efforts in bringing out this publication. We feel this compendium of student research articles will enlighten the young farm minds for better tomorrow in Indian Agriculture.

I deeply appreciate and acknowledge the Editorial Board, reviewers and scientists of R&D division of JEB for critical review and editing of the research papers. The co-operatation and assistance of secretarial and publication team of JEB in the completion of Special/Supplement issue is sincerely acknowledged. At last but not the least, I express my sincere thanks to Dr. R.C. Dalela for accepting the papers and considering the research papers for publication in *Journal of Environmental Biology*.

Original Research

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Effect of probiotic supplement feed on the foraging activity of Indian honeybee (*Apis cerana indica* F.)

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Abstract

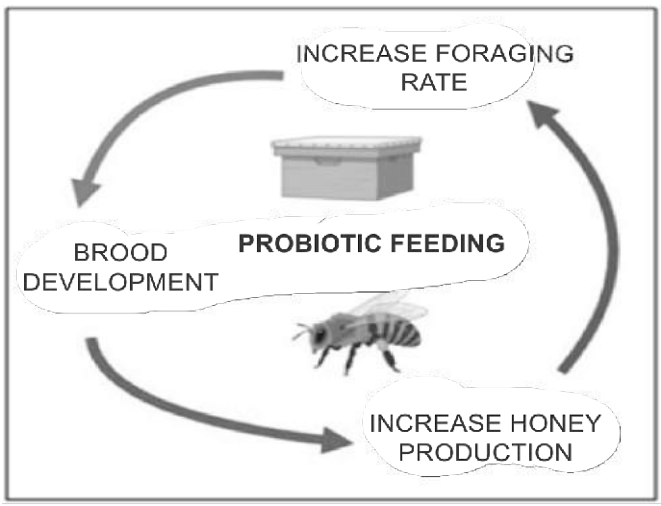
Aim: To investigate the effect of probiotic supplement feed on the foraging behaviour Indian honeybee colonies to resolve the excessive usage of antibiotics.

Methodology: The effect of sugar syrup feed (Untreated hives) and sugar syrup + probiotic supplement feed @ 10 ml 100 ml^{-1} syrup (Treated hives) on Indian honeybees was tested. The foraging activity and foraging rate were recorded and subjected to paired t- test analysis.

Results: The present observation showed a significant difference in the foraging rate between the colonies fed with sugar syrup + probiotic supplement (2.33 ± 0.11 min^{-1}) than the colonies that received sugar syrup alone (1.35 ± 0.06 min^{-1}). The highest foraging rate (2.80 ± 0.39 min^{-1}) was recorded in treated hives.

Interpretation: The experiment revealed that the brood development was directly proportional to brood pheromone levels, which positively affects the foragers, consequently foraging rate and honey production. Thus, the probiotic supplement feeding may influence the foraging behaviour of honeybee colonies.

Key words: Foraging rate, Honey, Indian honeybees, Probiotic supplement



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Introduction

The geographical position of India, and agro-climatic conditions supports the growth of a wide variety of cultivated and natural flora. Thus, an extended area of forest and cultivatable lands can sustain the bee keeping practices and honey production (Johnson *et al.,* 2010). Honeybees have many benefits other than income generation from honey production (Gupta *et al*., 2014). They are required for effective pollination of crops and are to improve the food production (Chantawannakul, 2018). Bee products are also considered as an important source of nutrition and have many medicinal applications (MusaOzcan and Juhaimi, 2015). Worldwide, 400 crop species are pollinated by honeybees (James and Pitts-Singer, 2008). Globally, 87 major food crops depend on animal pollination and they account for 35 per cent of the global food production (Van der Sluijs and Vaage, 2016). The crops pollinated by animals for reproduction are dependent on the managed honeybee for pollination (Breeze *et al.,* 2011). Hence, they play vital role for the economic, sustainable agriculture, food security, pollination of most wild flowers and maintaining the biodiversity.

These beneficial honeybees are often affected by various biotic and abiotic factors. Among the factors honey bee diseases caused by viruses, bacteria, fungi, protozoan and parasitic mites have been found to play a major role in increased honeybee mortality and collapse of the colony (Bailey, 1968; Kemp and Kross, 2000). Acaricides and antibiotics were used for the management of parasitic mites and diseases (Bogdanov, 2006). Among antibiotics oxytetracycline is commonly used to treat the bacteria *Paenibacillus larvae* and *Melissococcus plutonis*, the causal organisms of European foulbrood disease and American foulbrood disease, respectively (Johnson *et al.,* 2010). Intensive use of antibiotics, acaricides and other chemicals for management practices of diseases and parasites in bee keeping has consequently led to numerous side effects on the honeybees, residues in bee products, genotoxicity in consumers (Genersch, 2010).

Symbionts are microorganisms establishing interactions with their animal host, including insects and honeybees. They are involved in many aspects of host physiology, including nutrition, reproduction, immune homeostasis and defence (Sansonno, 2014). Understanding the interactions between the indigenous larval gut flora, nutrition and disease progression are important because the larval gut act as the target for many pathogenic bacteria and fungi (Vojvodic *et al.,* 2013). Thus, the indigenous gut bacteria play a role in withstanding the colonization of the gut by non-indigenous species, including pathogens (Dillion and Dillion, 2004). The gut microbiota modulation is considered a successful approach in the entomological field for the management of pest and pathogens (Alberoni *et al.,* 2018) and Gram-positive bacteria, such as lactic acid bacteriawhich are the promising options for pest and pathogen management in Apiculture (Audisio, 2017). According to the Food and Agriculture Organization of the United Nations and World Health Organization, probiotics are live microorganisms that confers health benefit to the host when administered in adequate amounts (FAO, 2001). Administration of different *Lactobacillus* and *Bifidobacterium* strains to infected larvae of *P. larvae*, significantly reduced their mortality (Forsgren *et al.,* 2010). Similar results were observed in the case of infection with the bacterial pathogen *M. plutonius* (Vasquez *et al.,* 2012; Wu *et al.,* 2014). Thus, this paper investigates the effect of probiotic supplement feeding on the foraging behaviour of Indian honeybee.

Materials and Methods

Field experiments were conducted in the Bee garden, Department of Plant Protection, Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirappalli district during 2018-19 to evaluate the effect of both sugar syrup feeding (Untreated hives) and sugar syrup + probiotic supplement feeding @ 10 ml $100ml^{-1}$ (Treated hives) on foraging activity of Indian honeybees . Each set of treatments had 10 bee hives kept at Bee garden with equidistant between each experimental bee hives. Foraging activity of bees were determined by counting the number of worker bees moving out and returning to the hive with and without the pollen loads by using hand tally counter and stop watch. The bees, carried pollen on their legs and empty foragers were noted separately. Bees returning without pollen loads were considered as non-pollen foragers/nectar gatherers. The foraging activity were recorded for three months during the period 2018-19 at fortnightly intervals and the observations were recorded three times a day *viz*., 8:00, 12:00 and 16:00 h for 5 minutes while the value obtained by sum of the three intervals were taken as the total foragers of the hive per 15 minutes for that particular day (Reddy *et al.,* 2015; Hemalatha *et al.,* 2018). The foraging rate was determined by counting the homing bees which were recorded three times a day *viz*., 8:00, 12:00 and 16:00 h for one minute. The mean of the values obtained at three intervals were taken as the foraging rate. The experimental data on the total foragers and foraging rate from the field experiments between treated and untreated were subjected to paired t- test analysis with Microsoft Excel using square root transformation ($\sqrt{x+0.5}$).

Observations on the number of honey cells and pollen cells stored in the brood frame were recorded for three months,during the period 2018-19 at weekly intervals using a transparent 1.0 cm grid in $100cm^2$ brood area (Delaplane*et al.,* 2013).The experimental data on the number of honey cells and pollen cells were also subjected to paired t- test analysis with Microsoft Excel using square root transformation ($\sqrt{x+0.5}$).

Results and Discussion

The foraging in honeybee is altruistic and socially regulated, but the individual nutritional physiology may also play a role in the foraging activity (Toth *et al.,* 2005). The foraging activity was highest in the colonies which received probiotic supplement feed. The foraging activity varied between both probiotic supplement-fed colonies and probiotic supplement non- fed colonies (29.60 ± 1.67 to 48.30 ± 2.45/15 min) (Table 1). The number of foraging bees that entered the hives were significantly highest ($P \leq 0.05$)

when probiotic supplement + sugar syrup was fed to the bees (48.30 ± 2.45/15 min) than in untreated hives (33.60 ± 1.63/15 min).

The foraging rate in terms of number of bees entering the hive per minute showed significant difference between those colonies fed with sugar syrup + probiotic supplement (2.33 ± 0.11/min) than in those colonies that received sugar syrup without probiotic supplement (1.35 ± 0.06/min) (Table 1). The highest foraging rate of 2.80 ± 0.39/min was recorded in treated hives. The present observations had a significant difference in the foraging behaviour between the treated and untreated hives.

Foraging efficiency of a colony was measured in terms of number of bees with pollen load entering into the hive. The foraging behaviours affected by both nectar and pollen, their quality and quantity (Pushpalatha, 2018). The pollen foraging bees that entered the hives ranged from 14.80 ± 0.73 to 20.70 ± 0.70/15 min in the colonies fed with probiotic supplement+ sugar syrup and sugar syrup without probiotic supplement (Table 2). The pollen foraging bees that entered the hives were highly significant ($P \leq 0.05$) when probiotic supplement + sugar syrup were fed to the bees (20.20 ± 0.38 per 15 min) and without probiotic supplement (15.83 ± 0.35 per 15 min). The probiotic supplements are involved in the health status and brood development of the honeybee colonies (Hamdi *et al.,* 2011; Alberoni *et al.,* 2016). The brood increase generates an

Table 1 : Effect of probiotic supplement feeding on the total number of foraging bees and foraging rate of Indian honeybee colonies

Month 2019	Fortnightly	Foraging bees per 15 min. (no.)			Foraging rate per min (no.)		
	count	Untreated hives	Treated hives	*t* – value	Untreated hives	Treated hives	*t* - value
March	I	31.20±1.64 (5.63)	46.60±1.62 (6.86)	6.41 (P=4.89E-6)**	1.33±0.17 (1.34)	2.10±0.23 (1.61)	2.32 (P=0.033)**
	II	33.60±1.04 (5.84)	47.60±2.42 (6.94)	5.18 (0.0002)**	1.60±0.27 (1.45)	2.80±0.39 (1.82)	2.14 (P=0.047)**
April	I	32.70±1.63 (5.76)	48.30±2.45 (6.99)	5.04 (P=0.0001)**	1.20±0.20 (1.30)	2.50±0.37 (1.73)	2.74 (P=0.016)**
	II	33.10±0.96 (5.80)	42.60±0.81 (6.57)	6.98 (P=2.24E-6)**	1.30±0.15 (1.34)	2.30±0.37 (1.67)	2.10 (P=0.056)**
May	I	30.40±1.35 (5.56)	47.90±1.94 (6.96)	7.16 (P=1.14E-6)**	1.40±0.22 (1.38)	2.20±0.20 (1.64)	2.30 (P=0.034)**
	II	29.60±1.67 (5.49)	46.50±1.47 (6.86)	7.19 (P=2.14E-6)**	1.30±0.15 (1.34)	2.10±0.28 (1.61)	1.99 (P=0.064)**
Mean		**31.77±0.65 (5.68)**	**46.58±0.85 (6.86)**	**13.84 (P=7.51E-8)****	**1.35±0.06 (1.36)**	**2.33±0.11 (1.68)**	**8.38 (P=3.11E-5)****

Mean ± SE; Mean of observations of 10 hives; Figures in parentheses are square root (√x+0.5) transformed values;* significant 0.01% level; **significant 0.05% level.

Table 2 : Effect of probiotic supplement feeding on pollen foraging bees and non-pollen foraging bees or nectar gatherers of Indian honeybee colonies

Month 2019	Fortnightly	Foraging bees per 15 min. (no.)			Foraging rate per min (no.)		
	Count	Untreated hives	Treated hives	*t* – value	Untreated hives	Treated hives	*t* - value
March	I	16.00±0.77 (4.06)	20.40±0.84 (4.57)	3.38 (P=0.003)**	23.20±0.61 (4.87)	28.50±0.50 (5.39)	5.97 (P=1.53E-5)**
	II	14.80±0.73 (3.91)	20.00±1.14 (4.53)	3.50 (P=0.003)**	22.90±0.57 (4.84)	27.30±0.61 (5.27)	4.66 (P=0.0002)**
April	I	17.20±0.88 (4.21)	20.70±0.70 (4.60)	2.70 (P=0.015)**	23.60±0.40 (4.91)	29.90±0.94 (5.51)	5.82 (P=5.93E-5)**
	II	16.40±0.65 (4.11)	20.00±0.77 (4.53)	3.06 (P=0.007)**	22.80±0.71 (4.83)	29.20±0.68 (5.45)	5.92 (P=1.34E-5)**
May	I	15.20±0.57 (3.99)	20.00±0.93 (4.54)	4.05 (P=0.001)**	22.10±0.69 (4.81)	29.80±0.92 (5.48)	6.39 (P=2.32E-5)**
	II						
Mean		**15.83±0.35 (4.04)**	**20.20±0.38 (4.55)**	**11.05 (3.27E-5)****	**22.87±0.21 (4.83)**	**29.03±0.40 (5.43)**	**5.97 (P=1.53E-5)****

Mean ± SE; Mean of observations of 10 hives; Figures in parentheses are square root (√x+0.5) transformed values; * significant 0.01% level; **significant 0.05% level.

Table 3 : Effect of probiotic supplement feeding on honey cells and pollen cells of Indian honeybee colonies

Month 2019	Fortnightly	Foraging bees of 15 min. (no.)			Foraging rate per min. (no.)		
	Count	Untreated hives	Treated hives	*t* – value	Untreated hives	Treated hives	*t* - value
	I	74.30±7.24 (8.65)	148.00±19.33 (12.19)	3.81 (P=0.002)**	24.30±2.92 (4.98)	43.90±4.62 (6.66)	3.70 (P=0.002)**
	II	95.70±13.16 (9.81)	145.30±16.75 (12.07)	2.36 (P=0.030)**	15.00±1.76 (3.94)	26.50±2.86 (5.20)	3.51 (P=0.003)**
March	III	84.80±21.70 (9.24)	151.20±22.80 (12.32)	2.38 (P=0.029)**	13.20±1.35 (3.70)	20.80±2.17 (4.62)	2.63 (P=0.018)**
	IV	65.20±7.58 (8.11)	144.60±31.99 (12.05)	2.46 (P=0.032)**	11.00±1.55 (3.39)	21.30±2.83 (4.67)	3.27 (P=0.004)**
	I	40.20±5.33 (6.38)	71.20±12.29 (8.47)	2.44 (P=0.027)**	8.00±0.77 (2.92)	16.40±1.66 (4.11)	4.73 (P=0.0002)**
	II	52.20±5.29 (7.26)	77.50±9.27 (8.83)	2.62 (P=0.019)**	18.20±2.08 (4.32)	29.00±0.94 (5.43)	4.33 (P=0.001)**
April	III	46.00±3.87 (6.82)	62.50±5.46 (7.94)	2.35 (P=0.031)**	17.20±2.59 (4.21)	26.50±2.68 (5.20)	2.72 (P=0.014)**
	IV	40.80±3.81 (6.43)	67.90±11.40 (8.27)	2.40 (P=0.032)**	13.10±1.31 (3.69)	20.40±2.17 (4.57)	2.64 (P=0.017)**
May	I	83.80±8.55 (9.18)	154.00±20.86 (12.43)	3.14 (P=0.007)**	11.90±1.27 (3.52)	21.30±2.83 (4.67)	3.21 (P=0.006)**
	II	89.40±11.69 (9.48)	153.20±25.74 (12.40)	2.36 (P=0.035)**	8.80±0.84 (3.05)	16.70±1.71 (4.15)	4.18 (P=0.001)**
Mean		**67.24±6.70 (8.23)**	**117.54±13.08 (10.86)**	**3.37 (P=0.004)****	**14.07±1.54 (3.82)**	**24.28±2.54 (4.98)**	**3.76 (P=0.002)****

Mean ± SE; Mean of observations at 10 hives; Figures in parentheses are square root (√x+0.5) transformed values; *significant 0.01% level; **significant 0.05% level.

expansion of bee colonies because of increase in the foragers. Moreover, a higher amount of pollen in this study is due to high foraging activity in honeybees which support both brood and bee health status (Di Pasquale *et al*., 2013).The number of non-pollen foraging bees per nectar gatherers that entered hives ranged from 22.10 ± 0.69 to 29.90 ± 0.94 per 15 min in the colonies fed with probiotic supplement + sugar syrup and sugar syrup without probiotic supplement (Table 2). The non-pollen foragers that entered the hives were significantly highest (P ≤0.05) when probiotic supplement + sugar syrup were fed to the bees (29.03 ± 0.40/15 min) than the probiotic supplement non-fed colonies (22.87 ± 0.21 per 15 min).

The number of honey filled cells recorded in treated hives ranged from 62.50±5.46 to 154.00±20.86 100cm^{-2} brood area and the number of honey cells recorded in untreated hives ranged from 40.20±5.33 to 95.70±13.16 100cm^{-2} brood area. The number of honey filled cells were highly significant in the colonies fed with probiotic supplement (117.54±13.08 100cm^{-2} brood area) (Table 3). The number of pollen/bee bread filled cells of *A. cerana* combs differed significantly between probiotic supplement fed hives (24.28±2.54 100cm^{-2} brood area) and probiotic non-fed hives (14.07±1.54 100cm^{-2} brood area) (Table 3). Comparatively the highest number of pollen cells (43.90±4.62 100cm^{-2} brood area) were recorded in the treated hives than the untreated hives.

The findings of this study showed that the pollen foragers (20.20 ± 0.38 per 15 min), non-pollen foragers (29.03 ± 0.40 per 15 min) and foraging rate (2.33 ± 0.11 per min) were high in treated hives which were fed with probiotic supplement feed than the unfed hives. Since the brood pheromone levels, was directly proportional to foraging behaviour which positively affects the number of foraging honeybees and their foraging rate, ultimately the honey production (Pankiw *et al*., 1998; Pankiw, 2004; Alberoni *et al*., 2018). Thus, they had influenced the foraging behaviour of the honeybee colonies and may influence the honey production since the number of foragers (46.58 ± 0.85 per 15 min) and honey cells (117.54±13.08 100cm brood area) were found to be more in treated hives.

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Add-on Information

Authors' contribution: I. Padmashree: Carried out research and drafted the manuscript; **S. S. J. Roseleen:** Major advisor of the research work; **C. G. L. Justin:** Supervised the research work and checked the manuscript.

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Effect of elevated temperature on the population dynamics of fall armyworm, *Spodoptera frugiperda*

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Abstract

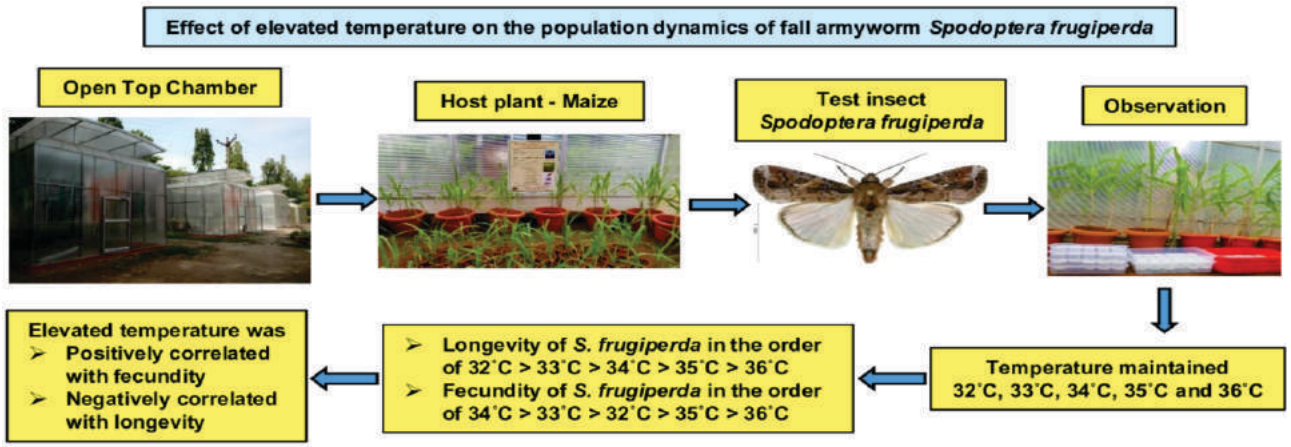
Aim: The present study was conducted to determine the growth, survival, fecundity and mortality rate of *Spodoptera frugiperda* in maize at elevated temperature.

Methodology: Biology and life table experiments were carried out in custom made Open Top Chambers (4m x 4m x 4m size) fitted with SCADA under increasing temperature regimes *viz.*, 32˚C, 33˚C, 34˚C, 35˚C and 36˚C. Each stage of the insects was examined daily and the fertility and life-table parameters were calculated by using TWOSEX-MS chart.

Results: The overall developmental time for egg, larva, pupa and adult stages (total lifespan) at different elevated temperatures were in the order of 32˚C > 33˚C > 34˚C > 35˚C > 36˚C. The fecundity rate of *S. frugiperda* at different elevated temperatures were in the order of 34˚C > 33˚C > 32˚C > 35˚C > 36˚C.

Interpretation: The current study confirms that the elevated temperature regimes had a positive correlation with fecundity rate upto 34˚C and negative correlation with the survival rate of *S. frugiperda.*

Key words: Life table, Population dynamics, *S. frugiperda*, Temperature



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Introduction

Insects flourish in all climates. Climate change, especially elevated CO_2 and elevated temperature, the effects insect physiological, behavioral, developmental process, species distribution and abundance. It was evidenced by changes in the number of generations per year, survival rate and earlier occurrence within the cropping season (Huang *et al.,* 2010). Invasive alien species (IAS) causes a serious threat to agriculture and cost billions of dollars in terms of reduced the productivity. About 1300 species of invasive insect pests and pathogens have been introduced into 124 countries due to increased trans-boundary movement of agricultural commodities, anthropogenic activities, climate change etc. (Paini *et al*., 2016).

Maize (*Zea mays*) the "Queen of cereals" is cultivated for human consumption, cattle and poultry feed. In India, maize is cultivated in an area of 93.80 million hectares with a production of 280.80 million tonnes and productivity of 3.07 tonnes per hectare. In Tamil Nadu, maize is cultivated over an area of 32.45 million hectares with a production of 259.17 million tonnes and 7.99 tonnes per hectare (Directorate of Economics and Statistics, 2018). Several insect pest causes damage to maize crop right from sowing to harvest, resulting in significant yield loss. Among them, the recently introduced pest, fall armyworm (*Spodoptera frugiperda*) is one of the serious concern due to its notorious and polyphagous behavior. *S. frugiperda*, is a native pest of America (Luginbill, 1928) and has recently been reported in Africa (Goergen *et al*., 2016) and India. First observations of *S. frugiperda* was made during 20l8 in Shivamogga, Karnataka (Sharanabasappa *et al*., 2018), consequently, reported in different parts of the country (Ganiger, 2018; Srikanth *et al*., 2018; Sisodiya *et al*., 2018 Bajracharya *et al*., 2019; Chormule *et al*., 2019). Larvae can survive on 353 different plant species belonging to 76 botanical families (Montezano *et al*., 2018). It is reported to cause major damage to the crops including rice, maize, sorghum, sugarcane, cabbage, beet, tomato, potato, cotton, peanut, soybean and alfalfa (Pogue, 2002; Chapman *et al*., 2000; CABI, 2016).

In ecological studies, life table is an important and necessary tool for analyzing and understanding the growth, survival, reproduction and mortality. Life table parameters help to identify the changes in the insect pests population during each developmental stage throughout their life cycle (Kakde et al., 2014). Different methods of analyzing life tables have been developed and widely adopted in ecological studies (Deevey, 1947; Birch, 1948; Southwood, 1978; Carey, 1993). The reproductive potential, fecundity and fertility of *S. frugiperda* have been studied under natural and controlled environmental conditions. This indicates a wide variation in those parameters, which may be influenced by temperature, larval diet and the strain of *S. frugiperda* (Simmons and Lynch, 1990; Rogers and Marti, 1994).

It is essential to understand the population dynamics of a pest before an effective IPM can be implemented (Maia *et al*., 2000). The traditional life table, which is based only on the female population (Birch, 1948). Age-stage, two-sex life table can eliminate many of the inherent errors characteristic of female-based life tables (Chi, 1988; Huang and Chi, 2011). Keeping in view the above, this study was conducted to determine the growth, survival, fecundity and mortality rate of *S. frugiperda* in maize at different elevated temperature levels using age-stage, two-sex life table.

Materials and Methods

Open Top Chamber: Open Top Chamber used in this study is a custom made cubical structure with a partially opened top side built with galvanized iron (GI) frames of 4 m x 4 m x 4 m and high-quality multilayer polycarbonate sheets (4-6 mm thickness). The chamber is provided with humidity and temperature supervising control and wireless signal transmission amenities along with Supervisory Control and Data Acquisition (SCADA) integration technology.

Host plants: The potted plants of maize (Variety: CO 6) were maintained using soil mixture of red soil + vermicompost + sandy soil in 1:1:1 ratio. Irrigation was done once in two days. From the day of sowing, the plants were maintained at different temperature inside open top chamber. These plants were used in the experiment as insect feed.

Insect source and rearing: Egg masses and larvae of *S. frugiperda* were collected from maize fields at Department of Millets in Tamil Nadu Agricultural University, Coimbatore. The collected insects were mass cultured in artificial diet (Patel *et al*., 1968) in laboratory of Department of Agricultural Entomology, Coimbatore for the availability of continuous and uniform culture of insects throughout the research period. Larvae were provided with artificial diet until larvae reached the pre-pupal stage. Following pupation, each pupa was kept in a separate plastic cup containing soil until eclosion. After eclosion, the adults were released into the rearing cages, covered with mesh net on three sides and glass on top, and a clean cotton wick containing 10% honey solution. The insects were bred for six complete generations under laboratory conditions (27±2˚C, 75±10% RH and 12L: 12D) before subjecting to life table studies at elevated temperature regimes.

Experiment design: Demography of *S. frugiperda* were studied at five different temperature regimes (32˚C, 33˚C, 34˚C, 35˚C and 36˚C) in separate Open Top Chambers (OTC). Single egg mass of almost equal size was taken per treatment. After hatching, the individual larvae were kept in separate plastic containers (3 cm diameter × 4 cm height). The larvae were fed with fresh maize leaves, it was changed every 24 hours, until pupation. Each individual was considered as separate replication. The number of replications depend on the number of eggs in individual egg mass taken at the beginning of the experiment and, hence, varied among five treatments. Data were collected daily, to monitor hatched eggs and the duration of each developmental stage of *S. frugiperda* on different elevated temperature levels. Dead larvae and malformed pupae were recorded and discarded every day.

After emergence, one female with one male were paired and kept in a mating cage. The fecundity rate was recorded daily from the period of oviposition until mortality of adults.

Life table study: For understanding the change in performance and population dynamics of insect pests on elevated temperature regimes, life table parameters are important indicators that explain the impact of growth, survival and fecundity of insect populations (Andrewartha and Birch, 1954; Chi and Su, 2006). Based on the procedures developed by Birch (1948) and Carey (1993), the following life table parameters *viz*., net reproductive rate (R_o), intrinsic rate of increase (r_m), finite rate of increase (λ), and mean generation time (T_c), gross reproductive rate (GRR) and population doubling time (DT) were calculated. The possibility of survival in function of age period of an insect pest followed Type III curve (logistic pattern) and displayed in semi-logarithmic scale (Gotelli, 2001). Therefore, fixation of survivorship curve was done using Doesn't Use Derivative (DUD) method (Raltson and Jenrich, 1978).

Statistical analysis: The age-stage, two-sex life table was used to analyse the raw life history and life table data of *S. frugiperda* using the computer program, TWOSEX-MS Chart (version 2019.11.17) (Chi 1988; Chi and Liu 1985). Data from the development time of *S. frugiperda* met the requirements of the analysis of variance (ANOVA) at $P < 0.05$, and their means were compared by Tukey's Honest Significant Difference (HSD) test using STAR (Statistical tool for agriculture research, IRRI, 2013). The population survival curve from the given sample can be estimated over different lengths of time period using Kaplan-Meier analysis in SPSS (Kaplan and Meier, 1958).

Results and Discussion

Insects are poikilothermic in nature, where their distribution range, growth, development, food consumption, number of generations and life cycle depends on atmospheric temperature conditions (Arora and Dhawan, 2013). In the present study, all the eggs hatched on 3rd day at 32˚C, 2nd day at 33 and 34˚C and 1st day at 35 and 36˚C. This result was on par with the findings of Du Plessis *et al*. (2020) on *S. frugiperda* in maize at different temperatures from 18 to 32˚C. Duration of all the larval stages were significantly different, for except L5 (fifth instar larva), when temperature increased from 32 to 36˚C. The total larval development was 11.71 days at 32˚C, whereas it was 9.16 days under 36˚C and the difference was statistically significant among the different temperatures. According to Ali *et al.* (1990), most of the *S. frugiperda* larvae completed their development in more than 6 stadia at 17°C, and all larvae died by fourth stadium at 38°C. Earlier, Mironidis and Savopoulou-Soultani (2014) found that the total larval developmental period of *H. armigera* in cotton was significantly longer with 68 days at 15˚C and it decreased to 14.52 days at 37.5˚C. The pupal stage also exhibited significant differences at different temperature regimes, with the shortest developmental time of 5.63 days at 36˚C and the longest of 10.16 days at 32˚C. Pupal eclosion was recorded at all the temperature regimes. Simmons (1993) recorded failure of eclosion in *S. frugiperda* at 10˚C and 40˚C, however, the eclosion was not affected between 15°C and 35°C. The duration of the pre-adult stage (egg to eclosion) also exhibited significant differences at different temperature regimes, with the shortest developmental time for the pre-adult stage occurring at 36˚C with 15.79 days and the longest at 32˚C with 24.88 days. The overall developmental time for egg, larva, pupa and pre-adult stages at different temperatures were in the order of 32˚C > 33˚C > 34˚C > 35˚C > 36˚C (Table 1). According to Du Plessis *et al*. (2020), the developmental time of *S. frugiperda* in maize was also decreased with increasing temperature regimes varying from 18˚C (34.39 days) to 32˚C (10.45 days) at larval stage and from 18˚C (30.68 days) to 32˚C (7.82 days) at pupal stage.

Adult longevity significantly decreased from 32˚C to 36˚C. The adult longevity at 32˚C was 11.02 days with male longevity and female longevity of 10.87 and 11.15 days, respectively. However, at 36˚C the male longevity and female longevity was 8.09 and 8.50 days, with adult longevity of 8.26 days. The order was 32˚C > 33˚C > 34˚C > 35˚C > 36˚C. Similarly, Mironidis (2014) reported that adult longevity of both sexes were significantly affected with fluctuating temperatures. The average life span of *H. armigera* male ranged from 9.88 to 20.39 days, whereas females from 11.09 to 18.56 days, at 32 and 25°C, respectively. The total lifespan significantly reduced increasing the temperature 32 to 36˚C. The mean total lifespan at from 32˚C was 35.90 days with 35.43 and 36.61 days for male and female entire lifespan, respectively. However, at 36˚C male and female entire lifespan was 24.18 and 23.88 days, respectively, with mean total lifespan of 24.05 days. The order of lifespan was 32˚C > 33˚C > 34˚C > 35˚C > 36˚C (Table 2). This result is in line with the susceptibility to elevated temperature studies. Tamiru *et al.* (2012) reported that at 22˚C the mean duration of *Chilo partellus* was 70.20 days while it needed only 26.50 days at 30˚C to complete its life duration.

The Adult Pre Oviposition Period (APOP) proved to be longest at 32˚C, with similar results found between 34˚C and 35˚C, following the order of 32˚C > 36˚C > 34˚C = 35˚C > 33˚C, while total pre oviposition period (TPOP) was longest at 32˚C, in the order of 32˚C > 33˚C > 34˚C > 35˚C > 36˚C. Females reared at 34˚C had the highest fecundity, while the lowest fecundity occurred at 36˚C, in the order of 34˚C > 33˚C > 32˚C > 35˚C > 36˚C and the difference was statistically significant. The number of oviposition days for females decreased from 34˚C to 36˚C, in the order of 34˚C > 32˚C > 33˚C > 35˚C > 36˚C and the difference was statistically significant (Table 3). Chen *et al*. (2014) reported that fecundity (eggs per female) increased with increasing temperatures in *Parapoynx crisonalis*. The number of eggs laid per female increased from 7.00 at 27˚C to 164.86 at 30˚C. Soh *et al*. (2018) revealed that the fertility per female of *Brevicoryne brassicae* was 39.84 nymphs per female at 15˚C but increased to 46.36 nymphs per female at 20˚C. Similar trend was also observed in this present study where the eggs per female was 1203.96 under 32˚C and steadily increased to 1805.58 at 34˚C.

Table 1: Developmental time (days) of *S. frugiperda* under different temperature regimes

Life stages	Temperature regimes										F	P
	32°C		33°C		34°C		35°C		36°C			
	n(N)	X ± SE	n(N)	X ± SE	n(N)	X ± SE	n(N)	X ± SE	n(N)	X ± SE		
Egg	106(123)	3.06±0.00^{a}	109(127)	2.08±0.00^{b}	103(128)	2.00±0.00^{b}	96(127)	1.25±0.00^{c}	81(125)	1.21±0.00^{c}	194.66	<0.000
L1	92(106)	1.76±0.05^{a}	89(109)	1.76±0.05^{ab}	87(103)	1.56±0.05^{bc}	74(96)	1.16±0.04^{c}	69(81)	1.12±0.04^{d}	13.98	<0.020
L2	78(92)	1.71±0.05^{a}	78(89)	1.65±0.05^{ab}	76(87)	1.55±0.06^{abc}	61(74)	1.25±0.05^{c}	54(69)	1.26±0.06^{bc}	2.97	<0.021
L3	66(78)	1.71±0.06^{a}	71(78)	1.68±0.06^{ab}	69(76)	1.43±0.06^{bc}	53(61)	1.26±0.06^{c}	47(54)	1.21±0.06^{c}	3.55	<0.008
L4	60(66)	1.73±0.06^{a}	63(71)	1.63±0.06^{a}	63(69)	1.48±0.06^{ab}	46(53)	1.28±0.07^{b}	38(47)	1.32±0.08^{ab}	2.57	<0.039
L5	54(60)	1.78±0.06	59(63)	1.78±0.05	62(63)	1.65±0.06	39(46)	1.54±0.08	32(38)	1.14±0.09	0.92	<0.455
L6	51(54)	2.75±0.07^{a}	54(59)	2.43±0.07^{b}	58(62)	2.14±0.07^{c}	36(39)	2.06±0.09^{c}	28(32)	1.61±0.09^{d}	22.51	<0.000
Total larva	51(106)	11.71±0.11^{a}	54(109)	11.12±0.11^{b}	58(103)	10.15±0.13^{c}	36(96)	9.53±0.20^{d}	28(81)	9.16±0.18^{d}	49.95	<0.000
Pupa	49(51)	10.16±0.09^{a}	52(54)	9.54±0.07^{b}	54(58)	8.54±0.09^{c}	32(36)	6.58±0.11^{d}	19(28)	5.63±0.14^{e}	310.83	<0.000
Pre-adult	49(123)	24.88±0.15^{a}	52(127)	22.65±0.13^{b}	54(128)	20.69±0.17^{c}	32(127)	17.09±0.24^{d}	19(125)	15.79±0.25^{e}	363.05	<0.000

N = total replicate number; n = effective replicate number; X = mean value; SE = standard error; F = F value of Tukey's Test; P = statistical significance. Means ± SE followed by different letters in the same row are significantly different at P < 0.05 (ANOVA followed by Tukey's Honest Significant Difference test)

Table 2: Adult longevity and lifespan of *S. frugiperda* under different temperature regimes

Parameters	Temperature regimes										F	P
	32°C		33°C		34°C		35°C		36°C			
(days)	n	X ± SE	n	X ± SE	n	X ± SE	n	X ± SE	n	X ± SE		
Male longevity	23	10.87±0.07^{a}	21	10.38±0.07^{a}	28	9.10±0.27^{b}	13	8.69±0.42^{bc}	11	8.09±0.11^{c}	12.26	< 0.000
Female longevity	26	11.15±0.10^{a}	31	11.06±0.09^{a}	33	10.88±0.12^{a}	19	9.89±0.14^{b}	8	8.50±0.13^{c}	27.50	< 0.000
Adults longevity	49	11.02±0.09^{a}	52	10.79±0.09^{a}	54	10.19±0.22^{b}	32	9.41±0.30^{c}	19	8.26±0.15^{d}	25.77	< 0.000
Male lifespan	23	35.43±0.15^{a}	21	33.33±0.14^{b}	28	30.05±0.31^{c}	13	26.62±0.43^{d}	11	24.18±0.25^{e}	119.87	< 0.000
Female lifespan	26	36.31±0.16^{a}	31	33.52±0.16^{b}	33	31.39±0.21^{c}	19	26.42±0.29^{d}	8	23.88±0.16^{e}	238.70	< 0.000
Total lifespan	49	35.90±0.16^{a}	25	33.44±0.15^{b}	54	30.87±0.26^{c}	52	26.50±0.34^{d}	19	24.05±0.21^{e}	325.12	< 0.000

n = effective replicate number;X = mean value; SE = standard error; F = F value of Tukey's Test; P = statistical significance. Means ± SE followed by different letters in the same row are significantly different at P < 0.05 (ANOVA followed by Tukey's Honest Significant Difference test)

Table 3: APOP, TPOP, oviposition period and fecundity of *S. frugiperda* under different temperature regimes

Parameter	Temperature regimes											
	32°C		33°C		34°C		35°C		36°C		FP	
	n	X ± SE	n	X ± SE	n	X ± SE	n	X ± SE	n	X ± SE		
APOP (days)	26	3.00± 0.00[a]	31	1.94±0.05[c]	33	2.00±0.00[c]	19	2.00±0.00[c]	8	2.12±0.13[b]	636.62	<0.000
TPOP (days)	26	28.15±0.21[a]	31	24.39±0.19[b]	33	22.52±0.23[c]	19	18.53±0.28[d]	8	17.50±0.39[d]	249.83	<0.000
Oviposition period (days)	26	6.27±0.09[b]	31	6.23±0.11[b]	33	7.39±0.14[a]	19	6.00±0.11[b]	8	4.75±0.16[c]	39.23	<0.000
Fecundity (Eggs/female)	26	1203.96±30.09[c]	31	1519.87±25.87[b]	33	1805.58±24.17[a]	19	784.21±17.73[d]	8	401±18.19[e]	308.90	<0.000

APOP = adult pre-ovipositional period; TPOP = total pre-ovipositional period (from egg to first oviposition); n = effective replicate number; X = mean value; SE = standard error; F = F value of Tukey's Test; P = statistical significance. Means ± SE followed by different letters in the same row are significantly different at P < 0.05 (ANOVA followed by Tukey's Honest Significant Difference test).

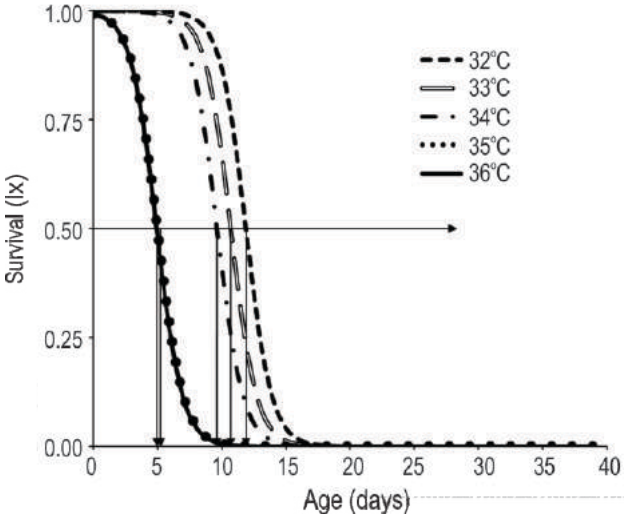

Fig. 1 : Survivorship curves of *S. frugiperda* under different temperature regimes.

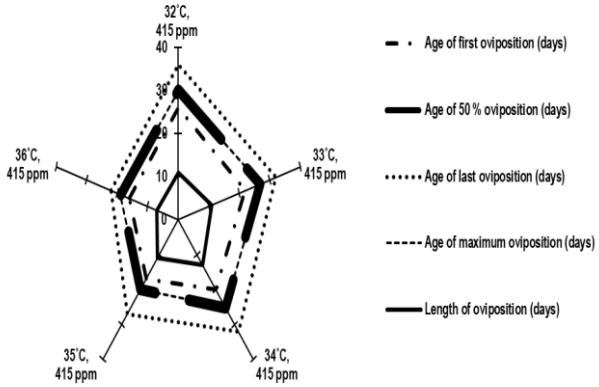

Fig. 2 : Radar representation of life characters of *S. frugiperda* under different temperature regimes.

The survival of *S. frugiperda* showed that it belongs to Type III survivorship curve (Fig. 1). The rate of mortality was found to be higher during early stage of the insect at higher temperature regimes (35 and 36˚C) as depicted by steep fall in the survival curve. The probability of survival of *S. frugiperda* is function of age trailed logistic pattern under increasing temperatures. The parameter 'a' (50 % mortality of the insect) symbolized the point of inflexion and the parameter 'b' symbolized the shape of the curve. The results showed that the survival per cent decreased with the function of age under increasing temperatures. At lower temperature (32˚C), 50 per cent mortality of *S. frugiperda* was observed after 11.90 days of development. However, at higher temperature regimes (35 and 36 ˚C), 50 per cent mortality occurred earlier on 5.30 and 4.90 days, respectively (Table 4). Similarly, the results of Golizadeh *et al.* (2007) revealed that increased temperature of 35˚C caused 100 per cent mortality in second instar larvae of *Plutella xylostella* in cauliflower. Kuo *et al*. (2006) studied the survivorship *of Rhopalosiphum maidis* in corn at seven different temperatures and found that 50 per cent mortality at 6˚C was observed on 38th day, while at 35˚C, 50 per cent and 100 per cent mortality was observed on 7th day and 14th day, respectively.

The life characters of *S. frugiperda* under elevated temperature are presented in Fig. 2. At 34˚C, the *S. frugiperda* started to oviposit at 20th day of pivotal age and it continued for the next 13 days and the maximum oviposition was recorded at 25th day of pivotal age. However, at higher temperatures (35˚C and 36˚C), the fertility started within 17th and 16th days by shortening its developmental period. At 35˚C and 36˚C, the length of oviposition observed was 11 and 7 days, respectively. The length of oviposition period decreased with increasing temperatures. This

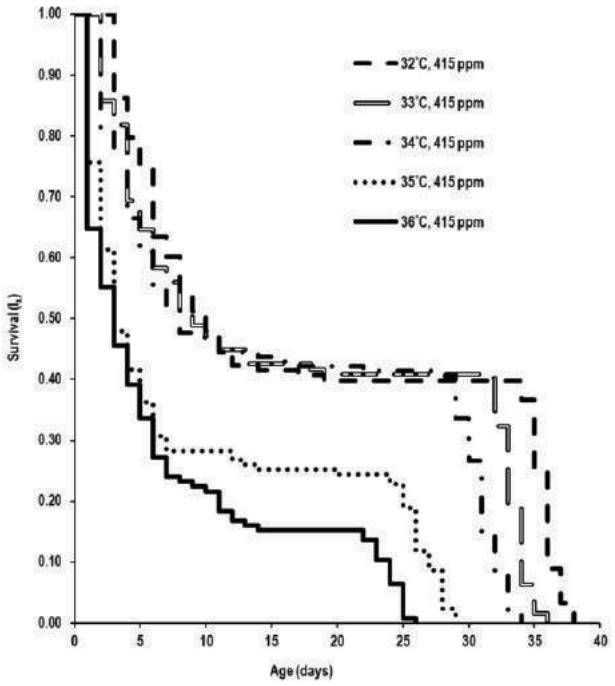

Fig. 3 : Kaplan-Meier estimate of survival of *S. frugiperda* under different temperature regimes.

signifies early maturity and the intense fertility of *S. frugiperda* under increasing temperatures.

The cumulative survival probability of *S. frugiperda* with respect to number of days in maize plants under elevated temperature levels are represented using Kaplan-Meier estimate in Fig. 3. Each survival curve indicated different treatments at elevated temperature levels. Each step down in each curve denotes the death of insects on each day. It was observed that the survival period of *S. frugiperda* ranged from 26 to 38 days at different treatments. The survival period of insect in different treatments were compared using log rank test. The mean survival distributions over 38 days was statistically non-significant between different treatments. The probability of insect mortality increased with increased temperature. Favored climatic conditions hastened the rate of growth and development, leading to more number of generations per year and hence probable expansion of geographical range (Parmesan *et al.*, 1999; Bale *et al.*, 2002).

The gross reproductive rate (GRR) of *S. frugiperda* observed was 645.61 number per female per lifetime at 32˚C and increased to 1212.66 number per female per lifetime at 34˚C whereas it was 163.16 number per female per lifetime at 36˚C. Similarly, the net reproductive rate (R_o) also increased from 254.62 number per female per lifetime (32˚C) to 473.82 numbers per female per lifetime (34˚C) and decreased at higher temperatures (112.67 and 24.82 numbers per female per lifetime at 35 and 36˚C, respectively). Soh *et al.* (2018) also observed similar trend in which the gross reproductive rate of *B. brassicae* was 27.31 at 10˚C and increased to 33.42 at 20˚C. The net reproductive rate increased from 10.23 nymphs per female at 10˚C to 24.04 nymphs per female at 20˚C. However, above 20˚C the net reproductive rate tend to decrease (11.06 and 0.15 nymphs per female at 25 and 30˚C, respectively). Ramalho *et al.* (2015) studied the life table parameters of *Hyadaphis foeniculi* in fennel at increasing temperatures of 20, 25, 28 and 30˚C. The results showed that the mean generation time decreased with increasing temperatures (23.51 days at 20˚C to 9.46 days at

Table 4: Response of survival of *S. frugiperda* under different temperature regimes

Temperature	Day at 50% mortality	Regression model	r^2 value
32˚C	11.90	y = -0.0185x + 0.5533	0.735
33˚C	10.80	y = -0.017x + 0.7656	0.727
34˚C	9.70	y = -0.0188x + 0.7683	0.766
35˚C	5.30	y = -0.018x + 0.5615	0.661
36˚C	4.90	y = -0.0218x + 0.5279	0.664

Table 5: Kinetics of life history of *S. frugiperda* under different temperature regimes

Parameters	Temperature regimes				
X ± SE	32°C	33°C	34°C	35°C	36°C
R_o	254.62 ± 46.77^c	374.39 ± 51.02^b	473.82 ± 68.43^a	112.67 ± 21.67^d	24.82 ± 7.84^e
r_m	0.18 ± 0.01^d	0.21 ± 0.01^c	0.24 ± 0.01^a	0.22 ± 0.01^b	0.16 ± 0.02^e
λ	1.19 ± 0.01^d	1.24 ± 0.01^c	1.27 ± 0.01^a	1.25 ± 0.01^b	1.17 ±0.02^e
T_c	31.12 ± 0.22^a	27.77 ± 0.21^b	26.09 ± 0.22^c	21.25 ± 0.29^d	20.10 ± 0.32^e
GRR	645.61 ± 85.53^c	924.89 ± 102.86^b	1212.66 ± 130.17^a	484.13 ± 70.48^d	163.13 ± 43.30^e
DT	3.90 ± 0.07^a	3.25 ± 0.12^b	2.95 ± 0.06^b	3.09 ± 0.11^b	4.28 ± 0.12^a

R_o = net reproductive rate (no./female/lifetime); r_m = intrinsic rate of increase (no./female/day); λ = finite rate of increase (no./female/day); T_c = mean generation time (days); GRR = gross reproductive rate (no./female/lifetime); DT = population doubling time (days); X = mean value; SE = standard error. Means ± SE followed by different letters in the same row are significantly different at P < 0.05 (ANOVA followed by Tukey's Honest Significant Difference

30˚C). The population doubling time also decreased with higher temperature regimes (11.09 days at 20˚C to 3.94 at 30˚C). Similar results were noted in the present study where mean generation time (T) at 32˚C was 31.12 days and it declined to 20.10 days at 36˚C. The population doubling time (DT) also reduced from 3.90 days (32˚C) to 2.95 days (34˚C). The intrinsic rate of natural increase (r_m) positively responded to elevated temperature conditions. At 32˚C, the r_m was 0.18 number per day and increased to 0.24 number per day at 34˚C. The same trend was also observed in finite rate of increase (λ) where it was 1.19 number per day at 32˚C and increased to 1.27 numbers per day at 34˚C (Table 5). Mironidis (2014) studied the effect of fluctuating temperature on *H. armigera* and found that r_m showed increasing trend from 0.077 individuals per day (17.5˚C) to 0.170 individuals per day (27.5˚C). At 17.5˚C, the finite rate of increase was 1.080 individuals per day and increased to 1.185 individuals per day at 27.5˚C.

In conclusion, the results of this indicate study that the elevated temperature levels showed positive correlation with fecundity rate and negative correlation with the survival rate of *S. frugiperda*. In this study, two-sex, age-stage life table of *S. frugiperda* 32, 33, 34, 35 and 36°C, and measured population parameters, including developmental duration, survival rate, fecundity, APOP, TPOP, r_m, λ, R_o, GRR, DT and mean generation time were established. The results indicated that *S. frugiperda* has strong reproductive potential at 34°C, and that this temperature range could result in rapid population growth. The present study demonstrated that it is vital to collect life table data at elevated temperature regimes for a comprehensive understanding of the effects of elevated temperature on pest populations and for predictive pest management.

Add-on Information

Authors' contribution: K. Ashok: Conducted the study, analysed, interpreted the data and wrote the first draft of the manuscript; **V. Balasubramani:** Designed the study, arranged funding, helped in interpreting the data, edited and proofread the manuscript; **: J. S. Kennedy:** Designed the study, helped in statistical analysis and revised the manuscript ; **V. Geethalakshmi:** Designed the study and arranged funding; **P. Jeyakumar:** Provided facilities for conducting experiments; **N. Sathiah:** Provided facilities for conducting experiments.

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Influence of elevated temperature on the fitness of Diamondback moth, *Plutella xylostella* (L.) in cauliflower

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Abstract

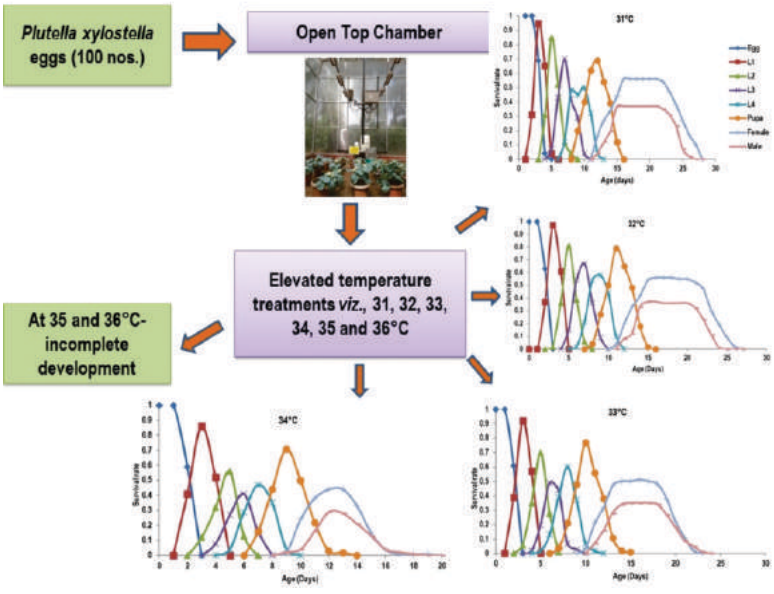
Aim: To understand the effect of elevated temperature on the bionomics and fitness parameters of *Plutella xylostella* which would help in predicting the population growth rates and formulating appropriate management tactics.

Methodology: In the present investigation, the fitness parameters of diamondback moth were studied at six different temperatures (31, 32, 33, 34, 35 and 36°C) in cauliflower in Open Top Chambers. Observations were recorded on the survival, longevity and fecundity at daily interval. The raw data on bionomics and life table parameters were analyzed using TWO-SEX- MS chart.

Results: The total life cycle of *P. xylostella* was longer at 31°C and it declined with increasing temperatures. However, *P. xylostella* did not complete its development at 35 and 36 °C. The intrinsic rate of increase (r_m) increased from 0.22 at 31°C to 0.28 at elevated temperature of 34°C. Temperature also had a significant effect on the net reproductive rate (R_0), Gross Reproductive Rate (GRR) and finite rate of increase (λ).

Interpretation: The fitness parameters will help to predict the change that occur in *P. xylostella* population due to climate change and global warming.

Key words: Diamondback moth, Life table, Population dynamics, Temperature



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Introduction

Diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is a devastating pest of cruciferous vegetable crops across the world (Garrad *et al.,* 2016, Jaleel *et al.,* 2019, Steinbach *et al.*, 2017). The yield loss due to this destructive insect pest is estimated to be around 50- 80% (Dhaliwal *et al.*, 2010). Globally, 4-5 billion US dollars have been spent on the management of *P. xylostella (Zalucki et al., 2012)*. The ability to complete number of generations per year, higher reproductive rate and insecticide resistance has made *P. xylostella* management difficult (Furlong *et al*., 2013, Gu *et al.*, 2010, Shelton and Wyman, 1992).

Insects are poikilothermic organisms, its growth, reproduction, survival and abundance are influenced by factors like temperature and photoperiod (Awmack and Leather, 2002, Malaquias *et al.*, 2010, Hallman and Denlinger, 1998). Thermal conditions required for the successful development of the pest vary in different ecological regions (Chen *et al.*, 2017, Gomi *et al*., 2003). Study of such variations are the need of the moment since global warming is likely to increase by 1.5°C between 2030 and 2052 (IPCC, 2018).

Precise knowledge of insect adaptations to changing climatic condition plays a vital role in effective management practices (Vargas *et al*., 2000). Life table parameters like intrinsic rate of increase (r_m) are important population measurement which helps to identify suitable Integrated Pest Management (Huang and Chi, 2013; Southwood and Henderson, 2000).

The effect of temperature on the biology of *P. xylostella* has been studied across the globe (Golizadeh *et al*., 2009; Liu *et al*., 2002; Shirai, 2000). However, most studies have been carried out under more extensive temperature variations, *i.e*., 10°C to 30°C. As we are impending towards the era of climate change, it is essential to know the demography of insect pests even with infinite simal temperature variations. Hence, in the present study, the fitness parameters of diamondback moth were studied on six sequential temperature treatments by using two-sex life table. Exploring the relationship between temperature and *P. xylostella* population will be useful for formulating accurate management practices in the future.

Materials and Methods

The seed of cauliflower (*Brassica oleracea* var. *botrytis*) variety Arka Kanti was grown in pots in a greenhouse and used for the experiments. The mother culture of *P. xylostella* larvae and pupae were collected from the cauliflower fields of Thondamuthur and Narasipuram, Coimbatore during February 2019. The collected larvae were reared on the leaves of cauliflower at 28 ± 2°C, 70 ± 5% RH in plastic containers (13 × 15 × 11 cm) covered with a fine muslin cloth. The pupae formed were placed in adult emergence cage (60 × 60 × 60 cm) which consisted clear, plexiglass on one side and remaining sides made of fine nylon mesh gauze. An opening was made on one side for providing fresh leaves and food. Honey solution (10%) was soaked in a cotton wick (10 cm) and given to adult as feed along with fresh cauliflower leaves (Golizadeh *et al*., 2009). The cage was covered with black muslin cloth for egg laying. Every 24h, the leaves with freshly laid eggs were collected from the cage and used for experiments.

A cubical open top chamber was built with high-quality multilayer polycarbonate sheets (4-6 mm thickness) partially opened top side 4 m x 4 m x 4 m made with galvanized iron (GI). The structure was outfitted with humidity and temperature supervising control and wireless signal transmission amenities along with SCADA (Supervisory Control and Data Acquisition) integration technology. Ceramic IR heaters could elevate the temperature around 10°C than control (ambient condition) autonomously in each OTC. Using SCADA system, a control signal from the controller was received by heaters which were a final control element (FCE). Different temperature regimes were attained correspondent to set temperature. Cauliflower plants were grown in pots for each temperature treatments in open top chambers.

Developmental parameters of *P. xylostella* were studied at six constant temperatures (31, 32, 33, 34, 35 and 36°C) in separate open top chambers. For studying the fitness parameters, ten *P. xylostella* eggs were collected from the surface of the leaf using small camel hair brush and placed on a leaf disc on water - soaked cotton in each 10 Petri dishes. A total of 100 eggs were examined at each temperature. A circular hole of 3 cm diameter fitted with fine nylon mesh was made on the lid for ventilation. The eggs were carefully examined until hatching. Each egg was noted as a replication. The newly emerged I instar larvae from each temperature treatment were placed individually on cauliflower leaf disc placed on water - soaked cotton in Petri dishes (4.5 cm dia, 4 cm ht). Fresh cauliflower leaves were given every day during the experimental period. The mortality, developmental time of each instar and life cycle of *P. xylostella* were recorded at each temperature treatment.

Fecundity and longevity of *P. xylostella* adults were examined at four constant temperatures (31, 32, 33 and 34°C). Temperature 35 and 36°C were excluded due to 100% mortality. Fifteen mating pairs were used for each treatment. Each pair was released in glass tubes covered with fine cloth. Crushed aluminium foil sheets swabbed with cauliflower leaf extract was provided as a substrate for oviposition. Egg laden foil sheets were replaced with new aluminium foil sheets every 24 hr and the number of eggs laid were counted until adults died.

The biological parameters of *P. xyllostella* were analyzed according to age- stage, two sex life table program TWO-SEX-MS chart (Chi, 2014). Age specific fecundity (m_x), age-specific survival rate (l_x) and population parameters were calculated according to Huang and Chi (2012). The intrinsic rate of increase (r_m) value was calculated as

$$r_m = log_e \frac{R_0}{T_c}$$

The *lx*, *mx* and R_0 vales are calculated using

$$l_x = \sum_{j=1}^{k} S_{xj}$$

$$m_{x=} \sum_{j=1}^{k} s_{xj} f_{xj} / \sum_{j=1}^{k} s_{xj}$$

$$R_0 = \sum_{x=\alpha}^{\beta} l_x m_x$$

The Gross Reproductive Rate (GRR) was calculated by the formula: GRR=$\sum m_x$. Mean separations of oviposition period and adult longevity were done by Duncan's Multiple Range Test (DMRT) using IBM SPSS v. 22 statistical program. Similar procedures were used for other parameters (r_m, λ, R_o, T, GRR and t).

The mean values of life table parameters were subjected to Bootstrap analysis (n=1,00,000) and the standard errors were estimated.

Results and Discussion

Life table is an important analytical tool to understand the growth, development and reproduction of insects under varying conditions. To get accurate estimates of fitness parameters, bootstrap technique was utilized with n= 1,00,000 and the means were used for calculating the standard errors (Huang and Chi, 2012). Insects can survive under a wide range of temperatures. The effect of temperature on the growth and development of various insects has been studied (Golizadeh *et al*., 2009; Manikandan *et al*., 2013; Chen *et al*., 2017; Soh *et al*., 2018).

P. xylostella completed its development at temperatures between 31 and 34°C. All the third instar larval stages died at 35°C whereas at 36°C, the eggs of *P. xylostella* did not hatch. The egg development time was significantly shorter at 35°C than other temperatures (P< 0.001).The duration of first, second, third and fourth instar larvae were significantly longer at the minimum temperature treatment of 31°C (1.99, 1.91, 2.07 and 2.03 days)

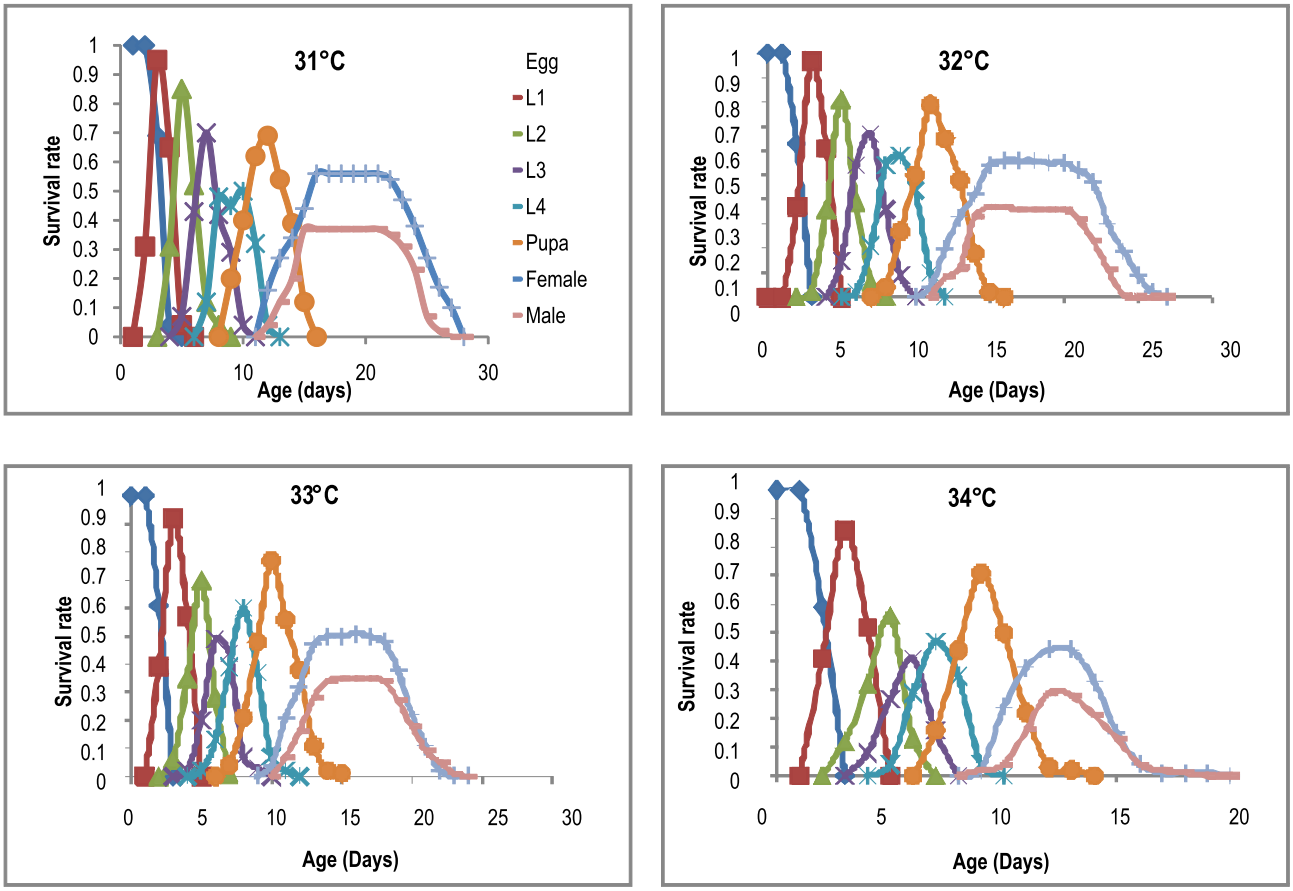

Fig 1. Influence of four different temperatures on the age – stage – specific survival rate (S_{xj}) of*Plutella xylostella*; L1 = 1st Instar, L2 = 2nd Instar, L3 = 3rd Instar, L4 = 4th Instar.

Table 1 : Influence of different temperatures on the biological traits of *Plutella xylostella* in cauliflower

Parameters	Temperature (°C)					F	P
(Days) (n=100)	31	32	33	34	35		
Egg	2.73 ± 0.05^{b}	2.61± 0.05^{b}	2.60 ± 0.04^{b}	2.63 ±0.05^{b}	2.56 ± 0.05^{a}	11.26	< 0.001
I instar	1.99 ± 0.01^{c}	1.97 ± 0.02^{c}	1.93 ± 0.03^{c}	1.86 ± 0.04^{b}	1.89 ± 0.04^{a}	3.208	< 0.001
II instar	1.91 ± 0.07^{e}	1.71 ± 0.07^{d}	1.50 ± 0.06^{c}	1.27 ± 0.05^{b}	1.35 ± 0.10^{a}	4.807	< 0.001
III instar	2.07 ± 0.06^{d}	1.91 ± 0.05^{d}	1.42 ± 0.06^{c}	1.13 ± 0.04^{b}	1.14 ± 0.14^{a}	3.829	< 0.001
IV instar	2.03 ± 0.05^{d}	2.04 ± 0.05^{d}	1.82 ± 0.05^{c}	1.44 ± 0.06^{b}	1.00 ± 0.01^{a}	4.251	< 0.001
Pupa	3.16 ± 0.05^{d}	3.12 ± 0.05^{d}	2.94 ± 0.06^{c}	2.67 ± 0.07^{b}	0.00 ± 0.00^{a}	4.603	< 0.001
Pre-adult	13.91 ± 0.14^{d}	13.39 ± 0.18^{c}	12.23 ± 0.13^{b}	10.93 ± 0.11^{a}	-	198.76	< 0.001

Means in the same row followed by same letter are not significantly different (P > 0.05) using bootstrap test.

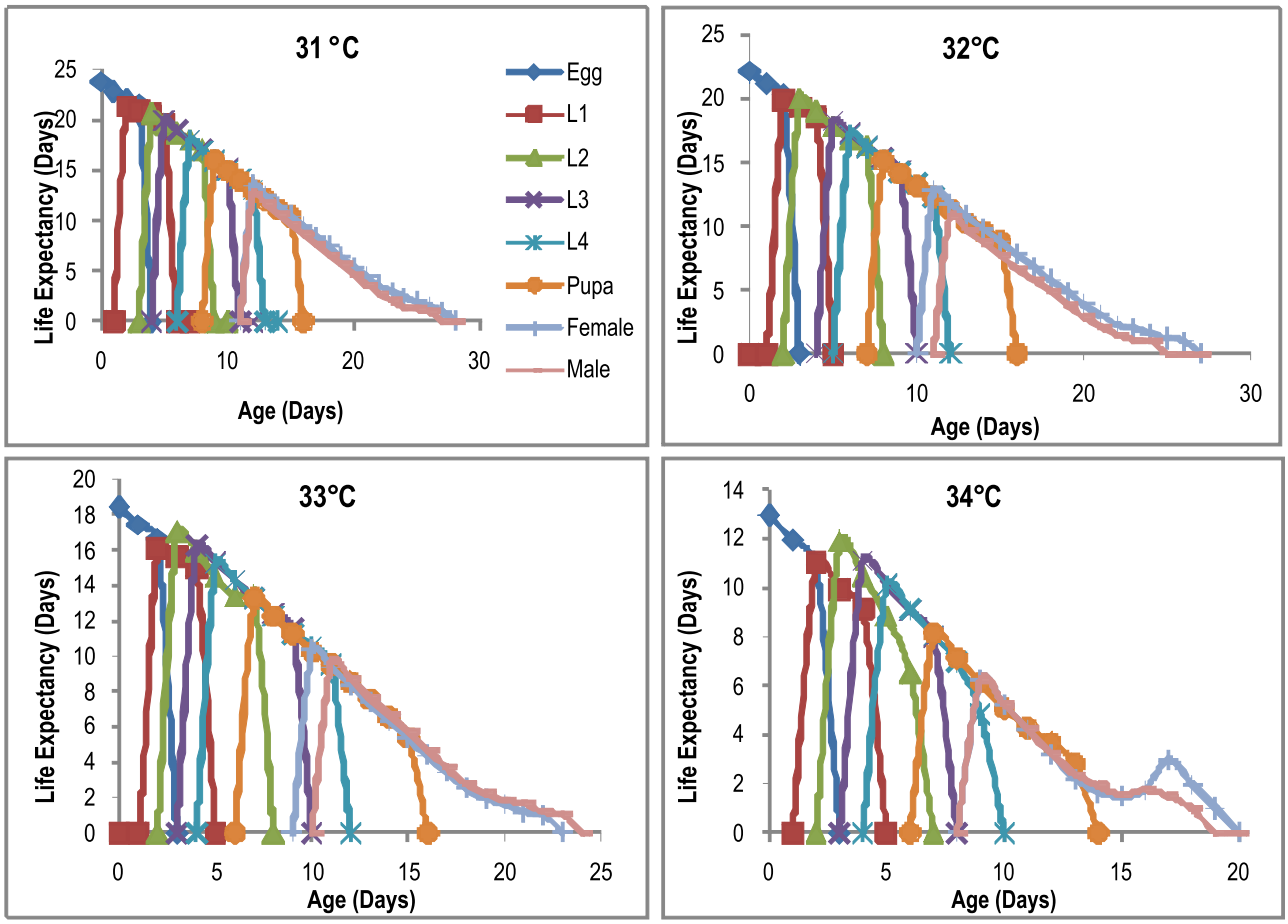

Fig. 2. Influence of different temperatures on age-stage-specific life expectancy (e_{xj}) of *Plutellaxylostella;* L1 = 1st Instar, L2 = 2nd Instar, L3 = 3rd Instar, L4 = 4th Instar.

and the duration steadily reduced at higher temperature of 35°C (1.89, 1.35, 1.14 and 1.00 days).The pupal duration lasted for about 3.16 ± 0.05 days at 31°C whereas it significantly reduced to 2.67 days at 34°C (P<0.001) (Table 1) (Fig. 1 and 2).

Similarly, in cabbage and cauliflower the survival rate of *P. xylostella* was reduced at 35°C (Golizadeh *et al*., 2009). Shirai (2000) found that 20- 25°C was the favorable condition for the reproduction of *P. xylostella,* while in our study the fecundity can be observed up to 34°C. Also, Liu *et al.* (2002) concluded that *P. xylostella* developed successfully from egg to adult stage at temperatures ranging from 8 - 32°C and there was only partial development at temperatures ranging from 34 - 40°C.

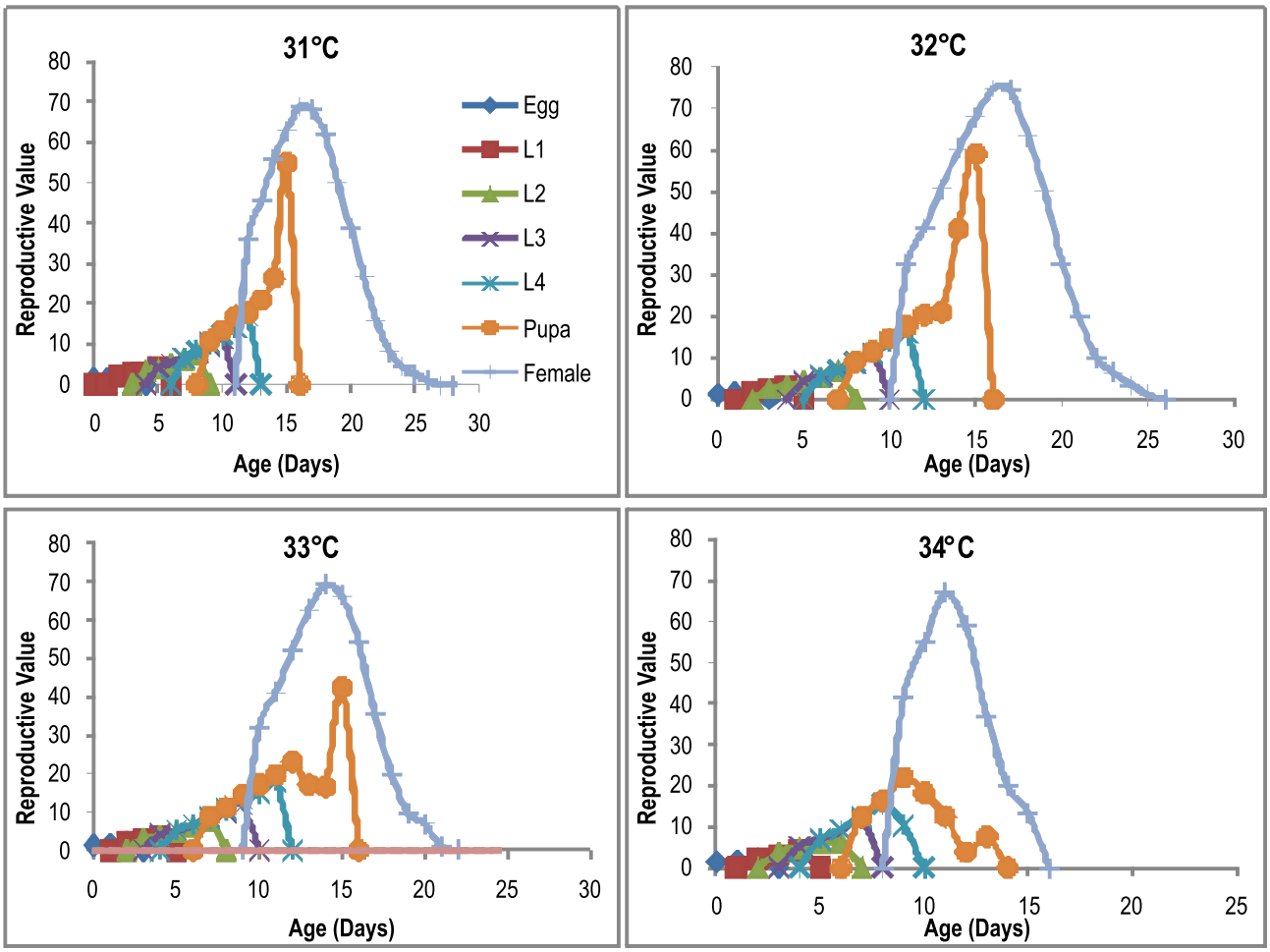

Fig. 3. Age-stage reproductive value (v_{xj}) of *Plutella xylostella;* L1 = 1st Instar, L2 = 2nd Instar, L3 = 3rd Instar, L4 = 4th Instar.

Table 2: Oviposition period of *Plutella xylostella* in cauliflower at different temperatures

Parameters	Temperature				F	P
(Days) (n=100)	31°C	32°C	33°C	34°C		
Male longevity	10.62 ± 0.14^{d}	9.14 ± 0.15^{c}	8.11 ± 0.10^{b}	3.87 ± 0.11^{a}	1649.30	< 0.001
Female longevity	11.61 ± 0.08^{d}	10.54 ± 0.13^{c}	8.35 ± 0.10^{b}	4.60 ± 0.09^{a}	447.70	< 0.001
APOP	1.73 ± 0.11^{c}	1.93 ± 0.12^{bc}	1.53 ± 1.33^{b}	0.87 ± 0.09^{a}	0.953	0.05
TPOP	15.6 ± 0.42^{d}	14.87 ± 0.39^{c}	13.66 ± 0.25^{b}	11.2 ± 0.22^{a}	6.085	< 0.001
Oviposition	8.6 ± 0.21^{d}	7.47 ± 0.16^{c}	5.66 ± 0.15^{b}	2.66 ± 0.15^{a}	1.084	0.399
Fecundity (total eggs/ female)	150.86 ± 19.32^{c}	142.80 ± 3.62^{bc}	117.07 ± 5.27^{ab}	100.33 ± 8.27^{a}	1.205	0.307

Means in the same row followed by same letter are not significantly different (P > 0.05) using bootstrap test.

Adult pre-oviposition period (APOP) did not vary significantly between the temperatures 31°C and 34°C (P= 0.514) and total pre-oviposition period (TPOP) ranged from 15.6 ± 0.42 days to 11.2 ± 0.22 days when the temperature increase from 31°C to 34°C, respectively (P< 0.001).The oviposition days of *P. xylostella* were inversely proportional with the increasing temperatures. At 31°C, the females oviposited for 8.6 days whereas it was decreased to 2.66 days at 34°C. Similarly, the fecundity reduced from 150.866 eggs per female at 31°C to 100.33 eggs per female at 34°C (Fig. 3).

The longevity of female is negatively correlated with the increasing temperatures. At 31°C, the longevity of female was 11.61 days and it significantly abridged to 4.60 days when the temperature was raised to 34°C. The maximum longevity of male was observed at 31°C (10.62 days) and minimum was at 34°C

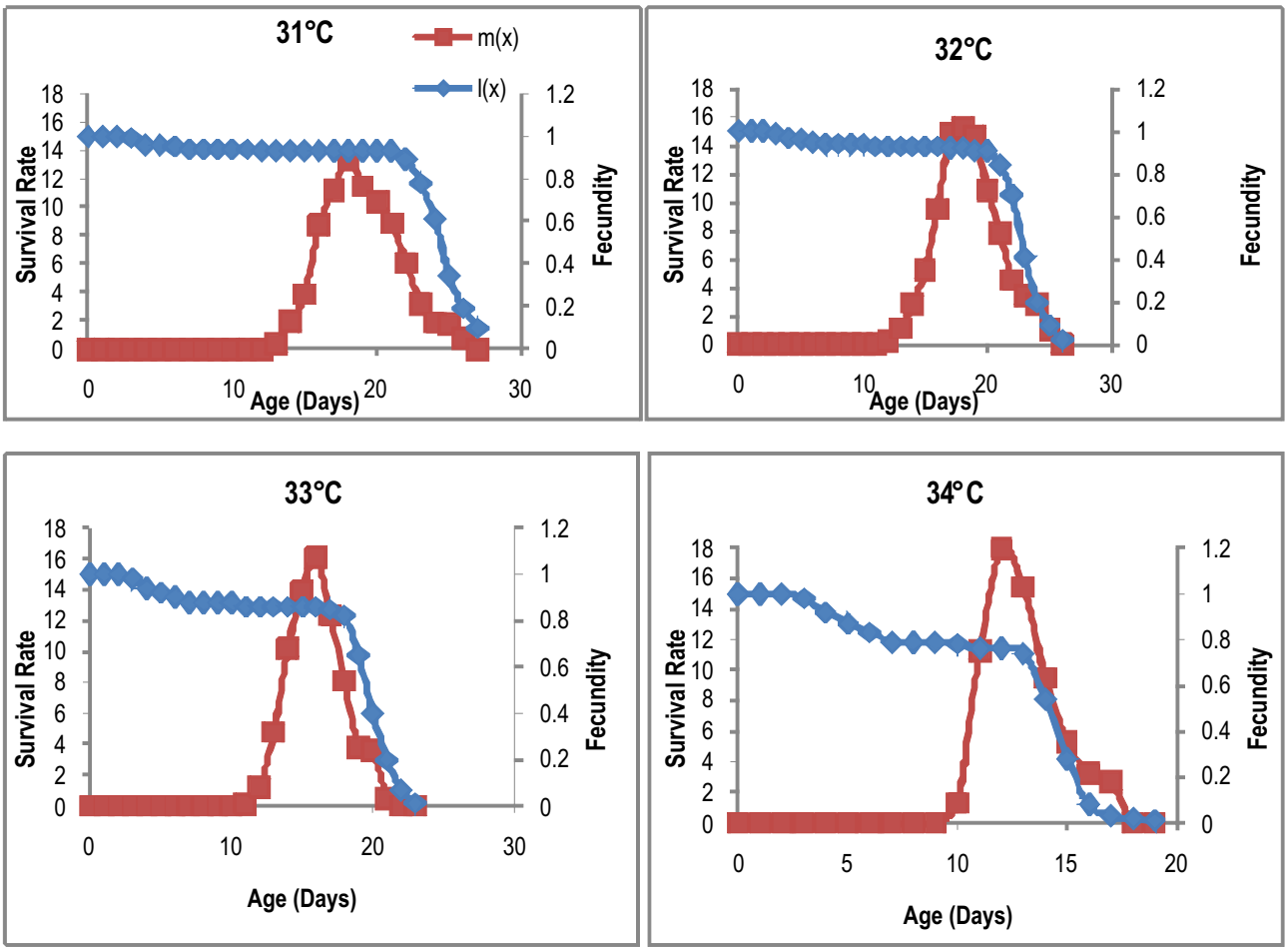

Fig 4: Influence of different temperatures on the survival rate (l_x) and fecundity (m_x) of *Plutella xylostella.*

Table 3: Influence of different temperatures on the life table parameters of *Plutella xylostella*

Parameters	Temperature				F	P
	31°C	32°C	33°C	34°C		
r_m	0.22 ± 0.001[a]	0.23 ± 0.002[a]	0.24 ± 0.002[b]	0.28 ± 0.007[c]	188.00	< 0.001
λ	1.25 ± 0.008[b]	1.27 ± 0.171[b]	1.28 ± 0.003[b]	0.32 ± 0.007[a]	4014.61	< 0.001
R_o	74.98 ± 0.737[c]	80.17 ± 1.355[d]	60.90 ± 0.507[b]	42.07 ± 1.007[a]	343.78	< 0.001
T	19.13 ± 0.038[c]	18.59 ± 0.377[c]	14.68 ± 0.122[b]	13.25 ± 0.324[a]	94.23	< 0.001
GRR	83.76 ± 0.179[c]	94.02 ± 1.663[d]	74.58 ± 1.474[b]	62.67 ± 1.501[a]	82.74	< 0.001
t	3.07 ± 0.010[d]	2.94 ± 0.016[c]	2.77 ± 0.015[b]	2.45 ± 0.038[a]	104.38	< 0.001

r_m: The intrinsic rate of increase (per day); λ: The finite rate of increase (per day); R_0: The net reproductive rate (offspring/individual); T: The mean generation time (days); t: Population doubling time; GRR: Gross reproductive rate (offspring); Means in the same row followed by the same letter are not significantly different (P > 0.05) using bootstrap test.

(3.87 days) (Table 2). There was an inverse relationship between temperature and age – specific survival rate (l_x) and fecundity (m_x) (Fig.4). Likewise, Garrad *et al*. (2016) found that *P. xylostella* reared at 30°C have shorter life span than insects reared at any other temperature, suggesting decreased fitness; however, they did produce a large number of eggs in the few days they were alive.

The fitness parameters of *P. xylostella* were notably affected by the increasing temperatures of 31°C to 34°C. The intrinsic rate of increase (r_m) had increased significantly from 0.22 to 0.28 when temperature increased from 31°C to 34°C (P< 0.001). Ngowi *et al.* (2017) showed that r_m value increased from 0.001 at 10°C to 0.20 at 30°C. It had been reported that the pre-adult and adult development rate varied at different temperatures (Folguera *et al*., 2010). The development time from an egg to adult, as well as the fecundity of insect pests attributed to the variations in r_m value (Badenes-Perez *et al*., 2005).

The net reproductive rate (R_0) was maximum with 80.14 individuals per female per lifetime at 32°C followed by 74.98 individuals per female per lifetime at 31°C and lowest R_0 value of 42.07 individuals per female per lifetime was observed at 34°C. The longest mean generation time was 19.13 days at 31°C compared to the shortest of 13.25 days at 34°C. The highest GRR value (94.02 numbers of offsprings per day) was found at 32°C; while lowest (62.67 numbers of offsprings per day) at 34°C (Table 3).

Jafary-Jahed *et al.* (2019) found that the net reproductive rate (R_0) of *P. xylostella* was 65.46 offsprings per individual when larvae were reared on cauliflower at 30°C, which nearly corroborated with the present finding where the net reproductive rate (R_0) was 74.98 individuals per female per lifetime at 31°C. The GRR is a measure of rapid increase of population that depends on the number of eggs laid, eggs hatched and adult eclosion, and all these parameters are exaggerated by temperature (Khaliq *et al*., 2007). In our study, the GRR decreased to 62.67 numbers of offsprings per day when the temperature increased to 34°C. The population increases only when net reproductive rate will be greater than 1 and r > 0 (Chen *et al.*, 2017, Southwood and Henderson, 2009). The findings of this study also confirmed the aforesaid theory. The information obtained from the present study can be used for developing forecasting models and formulating optimal integrated pest management strategies in the future era of global climate change.

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Add-on Information

Authors' contribution: K. Haripriya: Conduct of experiment, data recording and writing of manuscript; **J. S. Kennedy:** Conceptualization and supervision of research plan; **V. Geethalakshmi:** Review and finalization of paper, Supervision; **D. Rajabaskar:** Review and finalization of paper, Supervision.

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Modulations in carbon and nitrogen assimilation patterns in rice plants exposed to elevated atmospheric carbon dioxide concentrations

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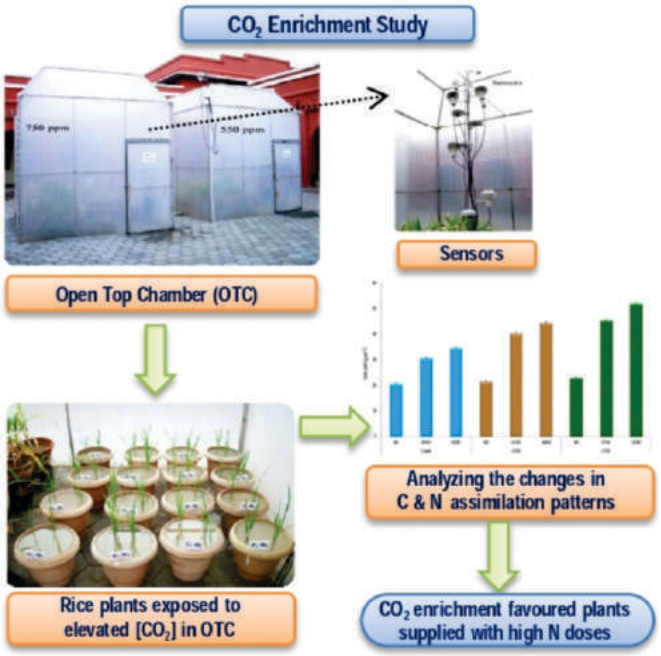
Abstract

Aim: To study the influence of elevated atmospheric CO_2 concentrations on the carbon and nitrogen assimilation patterns in rice plants.

Methodology: Rice (*Oryza sativa*) plants were placed in Open Top Chambers (OTCs) and exposed to elevated levels of CO_2. The treatments consisted of three levels of CO_2 (398, 550 and 750 µmol mol^{-1}) and three levels of nitrogen (0, 150 and 200 kg ha^{-1}) and replicated five times in completely randomized design.

Results: Leaf nitrogen was significantly reduced by 10.6 % and 6.5 % during later stages in rice plants exposed to CO_2 @ 750 µmol mol^{-1} and 550 µmol mol^{-1}, respectively over the ambient CO_2. Rice plants under elevated CO_2 did not exhibit any variations in Nitrate Reductase activity in leaves in comparison to ambient CO_2 at tillering stage. Interestingly, NRase activity in leaves decreased at flowering stage whereas NRase activity in roots increased at same stage. The highest mean nitrogen values (0.58, 0.89 and 1.35 %) were observed in C_{amb} (ambient CO_2 concentration) and the lowest values (0.51, 0.80 and 1.27 %) in C_{750} in roots, straw and grains, respectively. Elevated CO_2 @ 750 µmol mol^{-1} significantly increased the above ground biomass (straw and grain) by 15.6 and 40.1 %, respectively, over the ambient CO_2 of 398 µmol mol^{-1}.

Interpretation: Elevated CO_2 enhanced the grain productivity but affected the quality of rice grains. Thus, excessive nitrogen fertilization above the current recommendation is necessary for future high CO_2 environments.



Key words: Atmospheric CO_2 Open Top Chamber, Rice

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Introduction

Alarming increase in carbon emissions is a serious matter of concern for people on earth in general and environmental scientists in particular. Global warming is closely related to the rise in Green House Gas emissions such as CO_2, CH_4 and N_2O that contribute to the tune of 60, 20 and 6 % (IPCC, 2013). Despite several gases responsible for enhanced greenhouse effect, CO_2 emission is considered as a 'kingpin'. It has been observed that the atmospheric CO_2 concentration was 280 ppm in 1850 (Etheridge *et al.,* 1996) and increased to 411 ppm in the year 2019 (NOAA, 2019) that commensurate with mean temperature rise of 1.0°C during the 20th century (IPCC, 2018). This trend continues to increase at an alarming rate which may result in serious consequences. Agriculture is critical for human survival and its importance for the environment and climate is less recognized. The global concern of 21st century is food security to meet the demand of burgeoning population within the degrading natural resources (Arora *et al.,* 2011). Farming is further stressed by the impact of climate change. Rice (*Oryza sativa*) crop was selected because it is a staple food for more than half of the world's population (Bargali *et al*., 2007 & 2009) and grown on161 million ha with an average annual production of 6787 million tones (Vibhuti *et al*., 2015 a, b; Khatri *et al*., 2020; Negi *et al*., 2020). Rice continues to be a major food crop and serves as a staple diet for more than 700 million people in India (FAO, 2011). Despite the fact that the crop has been researched in various spheres focusing on productivity, the average per hectare yield hardly exceeds 5 tonnes. Moreover, the Carbon sequestration pattern of rice eco-system is likely to be altered or modified under the various scenarios of climate change. Such events are often predicted by enriching the atmosphere with CO_2 under Open Top Chamber conditions. It has also been estimated that the year 2050 and 2100 are the critical time frame for evaluation where atmospheric CO_2 concentrations is likely to exceed 550 and 750 ppm. It is well known that rice plant possesses C_3 photosynthetic pathway and is less efficient due to the fact that the assimilated carbon gets decarboxylated under the current atmospheric CO_2 concentrations. Thus, the elevated CO_2 is considered as carbon fertilization to promote photosynthetic activities and improve the productivity of crops. However, the other factors such as nitrogen fertilization, temperature and water may cause inhibitory or supplementary effects on carbon assimilation in rice plants. It is well documented beyond doubt that carbon and nitrogen cycles co-exist and complement each other (Reich *et al.,* 2006). Any alterations in one factor will up-regulate or down-regulate the biochemical pathways, but the mechanisms underlying such processes are poorly understood and need systematic investigations. Hence, this research was carried out to understand the influence of elevated atmospheric CO_2 concentrations on the carbon and nitrogen assimilation patterns in rice plants.

Materials and Methods

Open Top Chambers: The effect of elevated levels of CO_2 *viz.,* 550 µ mol mol^{-1} CO_2 and 750 µ mol mol^{-1} CO_2 on rice crops were investigated employing Open Top Chambers installed at the Department of Soil Science and Agricultural Chemistry, TNAU, Coimbatore. The equipment for monitoring and controlling the CO_2 in OTCs was fully automated and desired CO_2 concentrations (550 ppm and 750 ppm) were maintained throughout the experimental period. The entire system was linked to data logger and computer system with UPS for uninterrupted data recording and storing.

Microcosm study: Seven kilograms of wetland soil (air dried, 2mm) was weighed and transferred into a syntex pot (top diameter 18 cm, bottom diameter 16 cm, height 20 cm and the holes at the bottom were sealed for the purpose of water stagnation). FYM at the recommended dose of 12.5 t ha^{-1} (41.6 g pot^{-1}) and NPK at 150:50:60 kg ha^{-1} (500, 166.6, 200 mg NPK pot^{-1}) were applied in the form of urea, single super phosphate and muriate of potash, respectively. Zinc sulphate @ 25 kg ha^{-1} (83.3 mg pot^{-1}) was applied and was thoroughly mixed with the soil. Nitrogen and phosphorus were applied in four splits and P was applied basally before transplanting. Rice crop was treated with three different levels of Nitrogen *viz.,* 0, 150, 200 kg N ha^{-1} and the N was applied in four splits on soil weight basis. Nursery was raised in the wetland farm and 14 days old paddy (ADT 45) seedlings were transplanted into the pots. After establishment, two healthy seedlings were allowed to grow in each pot. Twenty five-day old rice crop was subjected to different CO_2 atmospheric conditions (398, 550 and 750 ppm µ mol mol^{-1} CO_2) and rice crop was continuously kept under these conditions until harvest. The pots were maintained under flooded conditions (cyclic submergence) throughout the crop period.

Treatments

Design: FCRD
Replications: Five

Factor 1:

C_{amb} - Ambient CO_2 concentration (398 µ mol mol^{-1} Co_2)
C_{550} - 550 µ mol mol^{-1} CO_2
C_{750} - 750 µ mol mol^{-1} Co_2

Factor 2:

N_0 - 0 kg N ha^{-1}
N_{100} - 150 kg N ha^{-1}
N_{200} - 200 kg N ha^{-1}

Initial soil characteristics: The soil used in the pot experiment was sandy clay and belonged to Noyyal series, classified taxonomically as *Typic Ustochrept* according to USDA classification. The results revealed that the soil was slightly alkaline in nature (pH =8.21) with low soluble salts (EC = 0.35). The soil was high in organic carbon content (6.78 g kg^{-1}), low in available nitrogen (110.3 mg kg^{-1}), medium in available phosphorus and potassium (6.8 mg $kg^{-1}$and 118.0 mg kg^{-1}), respectively.

Chlorophyll content: Fresh leaf samples (250 mg) were macerated in a pestle and mortar with 10 ml of 80 % acetone and centrifuged at 5000 rpm for 10 min. The supernatant was collected and the volume was made up to 25 ml using 80 % acetone and the chlorophyll content was obtained by measuring the OD at 645, 663 and 652 nm on a spectrophotometer (Elico BL 198) and expressed in mg g^{-1} fresh weight (Bruinsma, 1963).

Soluble proteins: Soluble proteins in rice leaves were determined by the Folin phenol method (Lowry et al., 1951) using bovine serum albumin (BSA) as a standard. The soluble protein content was expressed as mg g^{-1}.

Nitrate Reductase (Nrase): Nitrate reductase activity in leaves and roots were determined by adopting the method of Nicholas et al. (1976). The enzyme activity was expressed as µ mole NO_2 hr^{-1} g^{-1} fresh weight.

Glutamine Synthetase (GS): Glutamine synthetase activity in leaves and roots were determined by following method Sadasivam and Manickam (1991). GS activity was expressed as µmol γ-glutamyl hydroxamate formed per min per gram of fresh weight.

Glutamate Synthase (GOGAT): Glutamate synthase activity in leaves and roots were assayed by a modified method of Matoh et al. (1980) as described by Suzuki and Gadal (1982). One unit of GOGAT enzyme activity was expressed as 1 µmol of glutamate formed per min at 30°C.

Glutamate Dehydrogenase (GDH): Glutamate dehydrogenase activity in leaves and roots were assayes by the method of Sadasivam and Manickam (1991). The change in the absorbance at 340 nm was recorded and the GDH activity was expressed as µ mol NADH oxidized $min^{-1}g^{-1}$ fresh weight.

Carbon content: The carbon content of straw, root and grains were measured by using high temperature TOC analyser (Elementer, Model: Liqui TOC II). Temperature was maintained at 850°C pressure of 0.9 to 1 bar. Pure oxygen was used as a synthetic gas.

Nitrogen content: The nitrogen content of straw, root and grains were determined by method of Humphries (1956). About 10 mL of the diacid (H_2SO_4 and $HClO_4$ in 5:2) digest was taken and transferred to microkjeldahl to distill the Ammonia. 2% boric acid with 2-3 drops of double indicator was used to collect evolved Ammonia and titrated against 0.02 N sulphuric acid. Blanks were maintained without adding sample.

Statistical analysis: The data on various parameters studied during the course of investigation were statistically analyzed as suggested by Gomez and Gomez (1984). Wherever the treatment differences were found significant, critical difference were worked out at 5 % level of significance with mean separation by least significant difference and denoted by symbol * (** for 1%). Treatment differences that were not significant were denoted as 'NS'.

Results and Discussion

Physiological parameters such as total chlorophyll content, soluble protein and nitrogen assimilatory enzymes were estimated to gain insights on the response of rice plants under elevated CO_2 with varying levels of nitrogen. The total chlorophyll content was significantly higher under elevated CO_2 at the tillering stage, but the trend was reversed with the advancement of crop growth (Fig.1). The increased chlorophyll contents at the tillering stage under elevated CO_2 is due to the fact that the short-term CO_2 enrichment stimulates the rate of photosynthesis (Makino and Mae, 1999) and simultaneously increases the nitrogen uptake (Seneweera *et al.,* 2005). This statement is also supported by our results on leaf nitrogen content (Fig.2) which had positive correlation with chlorophyll content because during this period plant needs more nutrients for its physical growth. On the other hand, the decline in leaf chlorophyll in response to CO_2 enrichment cannot be solely explained by a dilution effect (Epron *et al.,* 1996). Such a decrease in leaf chlorophyll has frequently been reported in young plants in response to elevated CO_2 (Wullschleger *et al*., 1992) and may have some physiological significance. Decrease in chlorophyll content at the later stages under atmospheric CO_2 enrichment suggests that elevated CO_2 causes less Nitrogen to be invested in the light reactions of photosynthesis (Epron *et al.,* 1996). Further, many studies suggest that decreases in leaf Nitrogen and chlorophyll content under elevated CO_2 levels are often associated with reduced responses of leaf photosynthesis (Kim and You, 2010). In general, a decreasing trend in the chlorophyll contents was observed with the progression of crop growth irrespective of CO_2. This observation is line with Imai *et al.* (2008) who reported that the chlorophyll contents increased in young expanding leaves and decreased substantially during senescence. Nitrogen fertilization was found to increase the chlorophyll content irrespective of CO_2 or stages of observation. In any species, a large proportion of nitrogen is allocated to leaves throughout their life cycle. For instance, in rice, at early panicle initiation stage about 65-70 % of the total nitrogen in the shoot is invested in the leaf blade and 20 to 30 % in the leaf sheath (Seneweera et al., 2005). A major proportion of leaf N (~80 %) is allocated to chloroplasts and most of the nitrogen in the chloroplast are invested in photosynthetic protein, including large amount in Rubisco and Thylakoid Protein (Evans, 1989).

In this investigation, the soluble proteins were estimated (Table 1) as it accounts for more than 50 % of rubisco content (Suzuki et al., 2001) besides it is the reliable index for assessing the photosynthetic efficiency (Jensen and Bahr, 1977). The soluble proteins increased in the rice plants exposed to elevated CO_2 at the tillering stage, but the trend of response was reversed with the advancement of crop growth. Since soluble proteins are an indirect measure of rubisco content, this can be well explained with the literatures dealing rubisco content under elevated CO_2. It has been demonstrated that under elevated CO_2, rubisco content in the leaf varies during development (Seneweera et al., 2002) and it was found to be significantly reduced at the reproductive

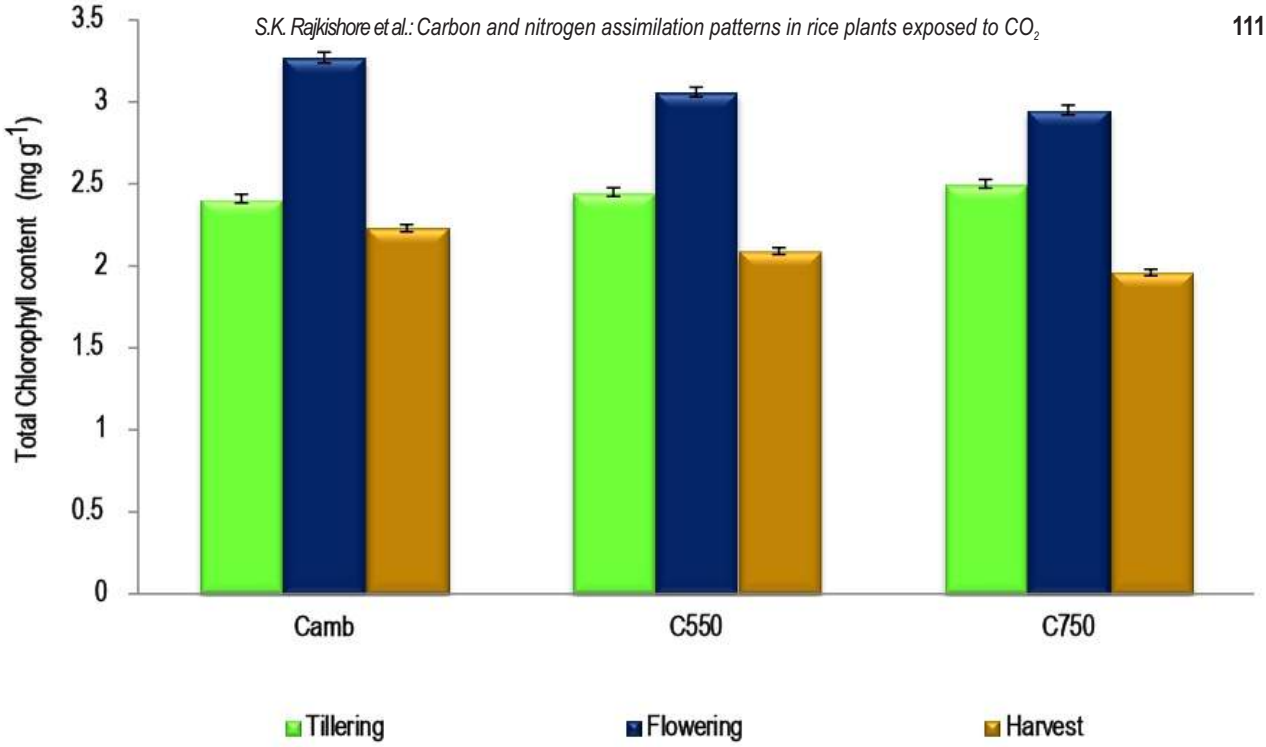

Fig. 1: Effect of elevated CO_2 on total chlorophyll content at various stages of rice growth.

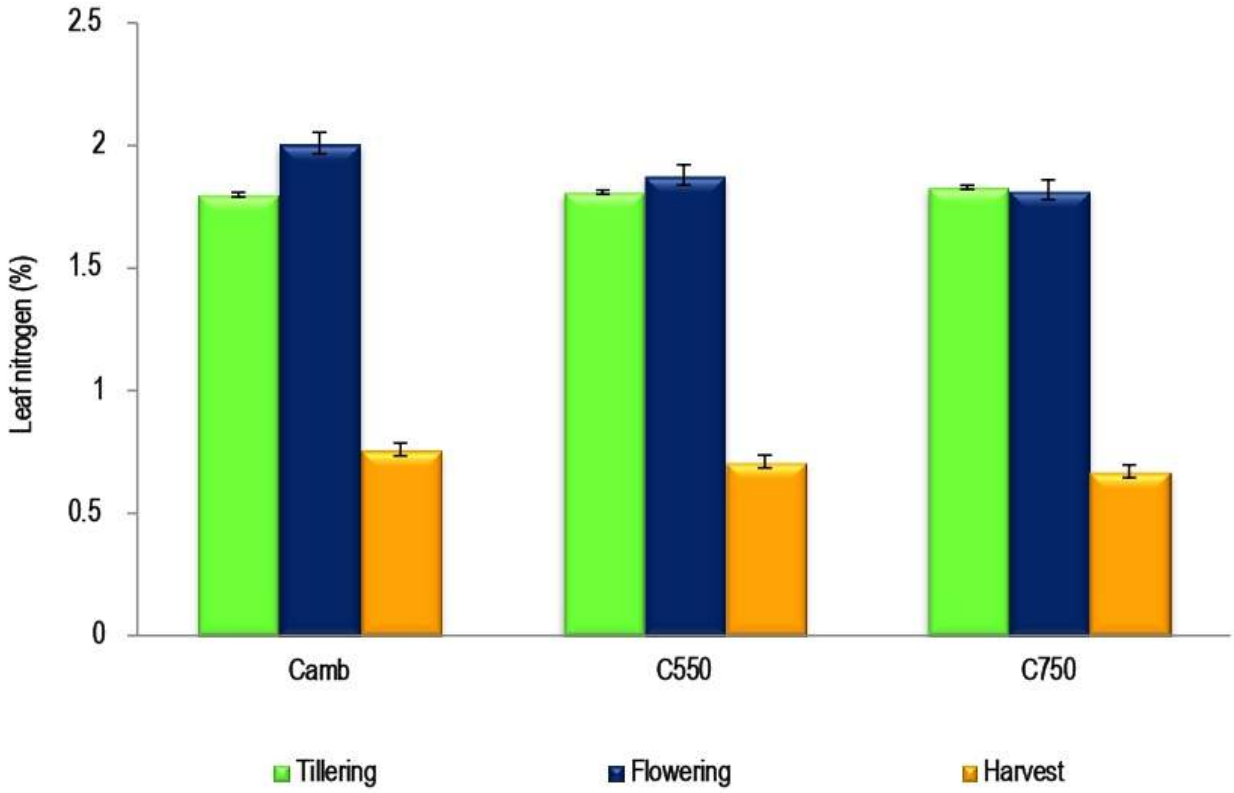

Fig. 2: Effect of elevated CO_2 on leaf nitrogen content at various stages of rice growth.

stage. These changes in rubisco content are related to modulation in C and N balance due to changes in source and sink activity during development but the underlying mechanism is not clearly understood (Seneweera et al., 2005). Another possibility is that plants reduced their N allocation for photosynthetic Proteins, making Nitrogen more freely available for rapid sink development. In the present study, even though the soluble proteins, an indirect measure of rubisco was found to be reduced at later stages under elevated CO_2, the biomass harvested is significantly higher under the elevated CO_2. This is attributed to the fact that at elevated CO_2, photosynthesis is not limited by rubisco content whereas electron transport capacity (Farquhar et al., 1980) or availability of P_i in the chloroplast to synthesize ATP limits the photosynthesis (Sharkey, 1985). This data suggest that rubisco content is not necessarily important to maintain high photosynthetic rates at elevated CO_2 and other regulatory mechanisms within the photosynthetic machinery may exist for downregulation of photosynthesis. Moreover, reports indicate that the decrease in rubisco content is not accompanied by a decline in CO_2 assimilation rates because the activation state of rubisco increased (Cheng and Fuchigami, 2000).

Our study indicated that on an average the leaf Nitrogen was significantly reduced by 10.6 % and 6.5 % during the later stages in the rice plants exposed to CO_2 @ 750 µmol mol^{-1} and 550 µmol mol^{-1}, respectively over the ambient CO_2. These results are in agreement with Anten et al. (2003) who reported that the rice grown at elevated CO_2 (200 µmol mol^{-1} above ambient) registered reduced leaf nitrogen. This decrease was found only after panicle initiation and the reason for these differences could be due to an increase in nitrogen demand for larger panicles in plants grown at elevated CO_2 (Seneweera et al., 2002). One of the common explanation for the reduction in leaf nitrogen concentration in plants grown at elevated CO_2 is the dilution of nitrogen due to extra Carbohydrate accumulation (Conroy, 1992). But several publications (Nakano et al. (1997); Makino and Mae, 1999) disagrees the 'dilution effect' concept and instead it is explained that the decrease in leaf nitrogen content is the result of a change in nitrogen allocation of the whole plant. According to Makino and Mae (1999), the nitrogen investment in the leaf blades decreases under CO_2 enrichment irrespective of nitrogen treatments, whereas the plants enhance the allocation of N to the leaf sheaths and roots. This means that during long-term growth under conditions of elevated CO_2, plants reallocate nitrogen away from leaf blades to leaf sheaths and roots. In addition, since nitrogen invested into leaf blades is the most important source for photosynthesis in the whole plant, these results also indicate that plants regulate photosynthesis by changing nitrogen allocation at the whole plant level.

To further understand the underlying reasons for reduced plant tissue Nitrogen under elevated CO_2, the nitrogen assimilatory enzymes *viz.,* Nitrate Reductase, Glutamine Synthetase, Glutamate Synthase, Glutamate Dehydrogenase in the rice leaves and roots were estimated (Table 2 and 3). The ability of plants to acquire and assimilate nitrogen is an important determinant of plant response to elevated CO_2 and of ecosystem carbon sequestration (Luo *et al*., 2004). In this study, the plants exposed to elevated CO_2 did not exhibit any variations in the NRase activity in leaves in comparison to ambient CO_2 at tillering stage. Interestingly, our data revealed that the NRase activity in leaves were decreased at flowering stage. This suggests that despite increased nitrogen uptake under elevated CO_2, the reallocation of nitrogen away from leaf blades might have led to decreased NRase activity in leaves at later stages of crop growth. Plant nitrogen status is a function of both soil N availability and plant nitrogen uptake and assimilation capacity. As a rate-limiting step in Nitrate assimilation, the reduction of Nitrate is an important component of plant physiological response to elevated CO_2 and terrestrial Carbon sequestration (Natali *et al.,* 2009). Both CO_2 and nitrogen enrichment had species specific impacts on NRase activity. Both above and belowground assimilation processes may be altered by increasing concentrations of atmospheric CO_2 (Searles and Bloom, 2003). The other possible reason for the reduction in NRase activity in leaves is that foliar NO_3^- reduction may compete for reductant with Calvin cycle reactions when Carbon assimilation is increased under elevated CO_2 (Bloom *et al.,* 2002). Because of the potential competitive effect between NO_3^- and CO_2 reduction, foliar NRase may decrease with CO_2 enrichment. However, variations in response are widely reported and this may be attributed to soil nitrogen form and concentration (Yong *et al.,* 2007), plant species/functional group (Cousins and Bloom, 2003) and altered diurnal rhythm of NRase activity (Geiger *et al*., 1998).

Table 1: Effect of elevated CO_2 and nitrogen levels on soluble protein contents

Treatments		Soluble protein content (mg g^{-1})					
		Tillering		Flowering		Maturity	
C_{amb}	N_0	7.5		13.6		4.2	
	N_{150}	8.4		17.9		6.1	
	N_{200}	9.0		18.7		6.5	
C_{550}	N_0	7.0		13.3		3.8	
	N_{150}	8.8		16.5		5.7	
	N_{200}	9.2		17.6		6.0	
C_{750}	N_0	6.7		12.8		3.4	
	N_{150}	9.1		16.0		5.5	
	N_{200}	9.4		17.0		5.8	
		SEd	CD	SEd	CD	SEd	CD
C		0.069	NS	0.131	0.267**	0.043	0.087**
N		0.069	0.139**	0.131	0.267**	0.043	0.087**
C x N		0.119	0.242**	0.228	NS	0.075	NS

CO_2 levels : C_{amb} - 398 µ mol mol^{-1} CO_2; C_{550} - 550 µ mol mol^{-1} CO_2; C_2 - 750 µ mol mol^{-1} CO_2; Nitrogen levels : N_0 – 0 kg N ha^{-1}; N_{150} – 150 kg N ha^{-1}; N_{200} – 200 kg N ha^{-1}; *$P \leq 0.05$, ** $P \leq 0.01$, NS - Non significant

Table 2: Effect of elevated CO_2 and nitrogen levels on Nitrate reductase and Glutamine synthetase activity in leaves

Treatments		Nitrate reductase (µmoles NO_2 g^{-1} hr^{-1})			Glutamine synthetase (µmol glutamyl hydroxomate min^{-1} g^{-1} f.wt)		
		Tillering	Flowering	Maturity	Tillering	Flowering	Maturity
C_{amb}	N_0	0.58	0.78	0.27	1.46	1.81	0.50
	N_{150}	0.66	1.01	0.39	1.63	2.33	0.73
	N_{200}	0.71	1.07	0.41	1.77	2.48	0.78
C_{550}	N_0	0.55	0.75	0.24	1.37	1.73	0.46
	N_{150}	0.69	0.93	0.36	1.72	2.16	0.69
	N_{200}	0.72	1.00	0.38	1.79	2.31	0.73
C_{750}	N_0	0.53	0.72	0.22	1.31	1.64	0.41
	N_{150}	0.71	0.91	0.35	1.78	2.10	0.66
	N_{200}	0.74	0.96	0.37	1.85	2.21	0.70

	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD
C	0.005	NS	0.007	0.014**	0.003	0.006**	0.013	NS	0.017	0.035**	0.005	0.011**
N	0.005	0.010**	0.007	0.014**	0.003	0.006**	0.013	0.027**	0.017	0.035**	0.005	0.011**
C x N	0.009	0.019**	0.012	NS	0.005	NS	0.023	0.047**	0.029	NS	0.009	NS

CO_2 levels : C_{amb} - 398 µ mol mol^{-1} CO_2; C_{550} - 550 µ mol mol^{-1} CO_2; C_2 - 750 µ mol mol^{-1} CO_2

Nitrogen levels : N_0 – 0 kg N ha^{-1}; N_{150} – 150 kg N ha^{-1}; N_{200} – 200 kg N ha^{-1}; *$P \leq 0.05$, ** $P \leq 0.01$, NS - Non significant

Table 3: Effect of elevated CO_2 and nitrogen levels on Glutamate Synthase and Glutamate dehydrogenase activity in leaves

Treatments		Glutamate Synthase (µmol glutamate min^{-1} g^{-1} fresh tissue)			Glutamate dehydrogenase (µmol NADH oxidized min^{-1} g^{-1} f.wt)		
		Tillering	Flowering	Maturity	Tillering	Flowering	Maturity
C_{amb}	N_0	2.33	2.77	0.89	0.69	1.03	0.35
	N_{150}	2.62	3.56	1.27	0.94	1.33	0.51
	N_{200}	2.75	3.79	1.39	0.97	1.41	0.55
C_{550}	N_0	2.19	2.65	0.78	0.66	0.99	0.32
	N_{150}	2.66	3.30	1.21	0.91	1.23	0.48
	N_{200}	2.85	3.52	1.30	0.95	1.31	0.51
C_{750}	N_0	2.10	2.56	0.73	0.61	0.95	0.29
	N_{150}	2.84	3.20	1.16	0.87	1.20	0.46
	N_{200}	2.92	3.39	1.26	0.93	1.26	0.49

	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD
C	0.021	NS	0.026	0.053**	0.009	0.019**	0.007	NS	0.009	0.019**	0.004	0.007**
N	0.021	0.043**	0.026	0.053**	0.009	0.019**	0.007	0.014**	0.009	0.019**	0.004	0.007**
C x N	0.037	0.075**	0.045	NS	0.016	NS	0.012	0.025**	0.016	NS	0.006	NS

CO_2 levels : C_{amb} - 398 µ mol mol^{-1} CO_2; C_{550} - 550 µ mol mol^{-1} CO_2; C_2 - 750 µ mol mol^{-1} CO_2

Nitrogen levels : N_0 – 0 kg N ha^{-1}; N_{150} – 150 kg N ha^{-1}; N_{200} – 200 kg N ha^{-1}; *$P \leq 0.05$, ** $P \leq 0.01$, NS - Non significant

Carbon metabolism is inextricably linked to Nitrogen metabolism and any change in Carbon abundance impacts on Nitrogen metabolism and vice versa (Lewis *et al.,* 2000). There is clear evidence that more than 95 % of the Ammonium available to higher plants is assimilated *via* the GS/GOGAT pathway (Lea and Miflin, 1974). In the present study, the trend of response for GS, GOGAT in rice leaves were similar to the NRase activity. The GS and GOGAT activities of leaves have shown to be lower under elevated CO_2 indicating the process of remobilization of metabolites towards reproductive organs which in turn support grain growth. Moreover, the glutamate is the precursor for chlorophyll synthesis in leaves (Yaronskaya *et al.,* 2006) and

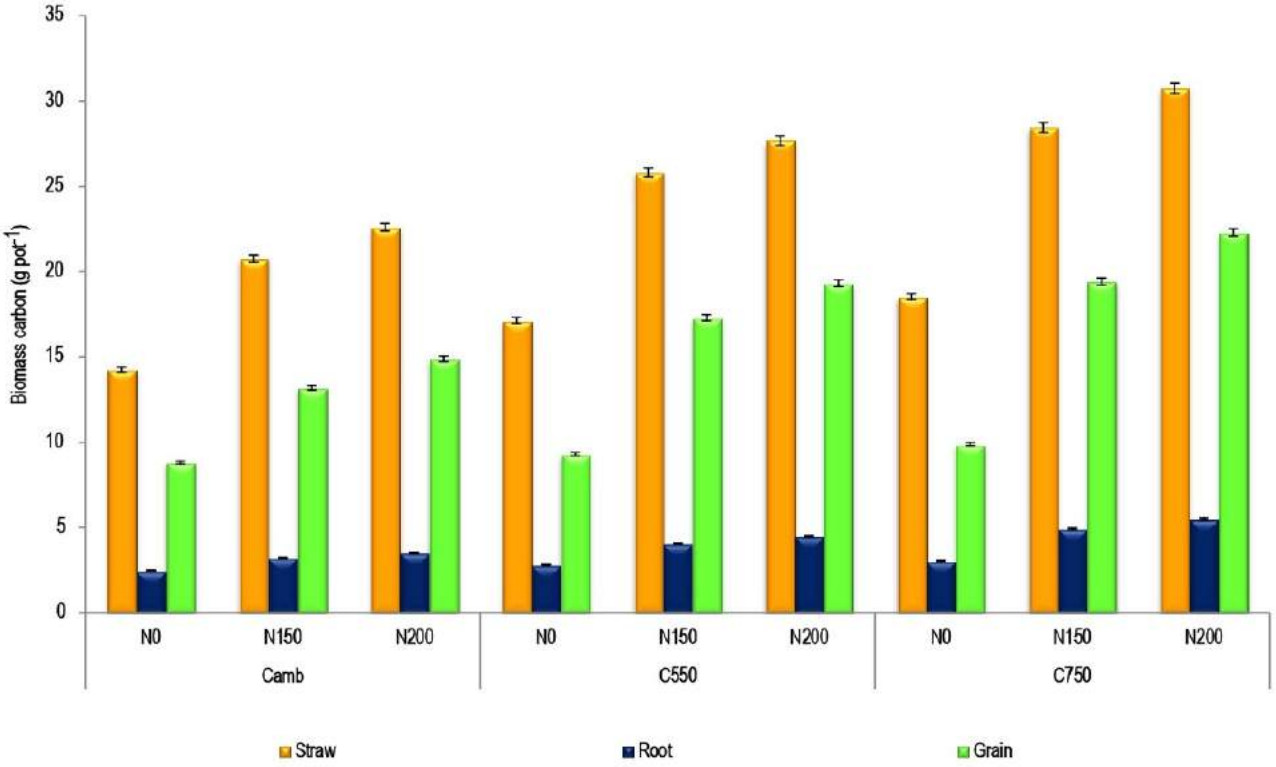

Fig. 3: Effect of elevated CO_2 and different levels of nitrogen on rice biomass carbon.

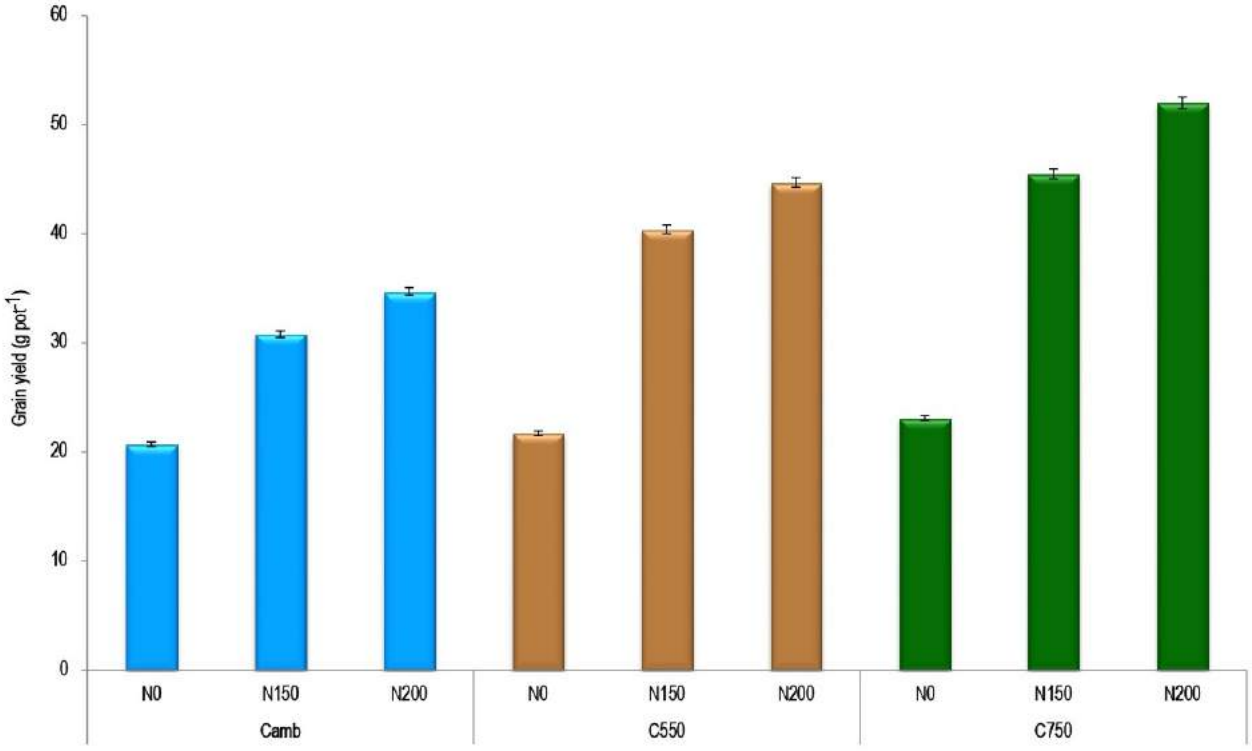

Fig. 4: Effect of elevated CO_2 on different levels of nitrogen on rice grain yield.

Table 4: Effect of elevated CO_2 and nitrogen levels on yield

Treatments		Grain yield (g per pot)		Straw yield (g per pot)		Root yield (g pot^{-1})		Root : Shoot ratio	
	N_0	20.7		40.5		5.9		0.146	
C_{amb}	N_{150}	30.8		59.1		7.7		0.130	
	N_{200}	34.7		64.3		8.4		0.131	
	N_0	21.7		43.5		7.3		0.168	
C_{550}	N_{150}	40.4		65.4		10.5		0.161	
	N_{200}	44.7		70.1		11.6		0.165	
	N_0	23.1		45.2		7.7		0.170	
C_{750}	N_{150}	45.5		69.2		12.4		0.179	
	N_{200}	52.0		75.0		13.8		0.184	
		SEd	CD	SEd	CD	SEd	CD	SEd	CD
C		0.21	0.43**	0.35	0.71**	0.63	1.28**	0.001	0.002**
N		0.21	0.43**	0.35	0.71**	0.63	1.28**	0.001	0.002*
C x N		0.36	0.74**	0.60	1.22**	1.09	NS	0.002	0.003**

CO_2 levels : C_{amb} - 398 µ mol mol^{-1} CO_2; C_{550} - 550 µ mol mol^{-1} CO_2; C_2 - 750 µ mol mol^{-1} CO_2

Nitrogen levels : N_0 – 0 kg N ha^{-1}; N_{150} – 150 kg N ha^{-1}; N_{200} – 200 kg N ha^{-1}; *$P \leq 0.05$, ** $P \leq 0.01$, NS - Non significant

therefore the positive correlation of chlorophyll contents in leaves with GS and GOGAT activities can be taken as a support for our interpretation.

The carbon sequestration potential in terms of above ground and below ground biomasses of rice crops exposed to elevated CO_2 at varying levels of Nitrogen were assessed. The data demonstrated that elevated CO_2 @ 750 µmol mol^{-1} significantly increased the above ground biomass (straw and grain) by 15.6 and 40.1 %, respectively, over the ambient CO_2 of 398 µmol mol^{-1}. Interestingly, the rice plants under elevated CO_2 of 750 µmol mol^{-1} largely increased their below ground biomass (root) by 54.8 % over the ambient CO_2 (Table 4). In the case of rice plants exposed to elevated CO_2 @ 550 µmol mol^{-1}, the above ground (straw and grain) and below ground biomass (root) were significantly increased (9.3 and 24.0, 34.2 %), respectively over the ambient CO_2. These results are in agreement with previous experiments (Kim *et al.,* 2003; Baker, 2004; Krishnan *et al.,* 2007; Madan et al., 2012) and modeling studies (Bannayan *et al.*, 2005) that reported enhanced rice biomass as a result of CO_2 enrichment. The increase in aboveground biomass with elevated CO_2 is mainly attributed to increased photosynthesis and tiller number (Sakai *et al.,* 2001). Growth during the seedling stage is 'source-limited' and the Carbohydrates increased by CO_2 enrichment are efficiently utilized for additional sink such as the development of new tillers or secondary shoots (Makino *et al.,*1997). Further, the increased below ground biomass is as a result of increased C allocation to the belowground, changes in root morphology (Kim *et al.,* 2001). In this study, the root/shoot ratio of rice plants under elevated CO_2 (750 and 550 µmol mol^{-1}) increased 30.9 and 21.3 %, respectively over the ambient CO_2. This data is in agreement with Uprety et al. (2000) and Gai-ping *et al.* (2006) who reported that rice root/shoot ratio was significantly increased by 155 and 35 % under CO_2 enrichment in comparison to ambient treatment. The increase in root/shoot ratio may be due to redistribution of carbon in plant tissues since more carbon can be allocated to the roots under elevated CO_2 concentrations (Gorissen and Cotrufo, 2000).

In the present investigation, the Nitrogen fertilization was a crucial factor for tapping the carbon sequestration potential of rice plants under higher levels of CO_2. Previous studies (Ziska *et al.,* 1996; Seneweera *et al.,* 2005; Kim *et al.,* 2011) have unequivocally demonstrated that the highest Nitrogen doses are pivotal for rice plants to harness the potentials of 'CO_2 fertilization effect'. So far the interactive effect of Carbon and Nitrogen on growth has mainly been attributed to two mechanisms. First, inhibition of leaf photosynthesis by Carbohydrate accumulation after prolonged exposure to elevated CO_2 tends to be stronger under low than under high nitrogen availability (Rogers et al., 1996). Second, nitrogen uptake increases under elevated CO_2 only when nitrogen availability is high (Stitt and Krapp, 1999). Numerous experiments have demonstrated that elevated CO_2 greatly increased dry matter accumulation of rice, with the magnitude of the response depending on nitrogen level (Shimono and Bunce, 2009).

Our observations revealed that the elevated levels of CO_2 not only enhanced the total biomass of the rice plants but also increased the carbon content of rice roots and straw (Table 5). However, the Carbon content in grains was unaltered. Rice plants exposed to elevated CO_2 of 750 and 550 µmol mol^{-1} registered an increase in the Carbon contents of roots (8.5 and 2.6 %) and straw (16.5 and 12.2 %) over the ambient CO_2. These results are in

Table 5: Effect of elevated CO_2 and nitrogen levels on carbon content, nitrogen content and nitrogen uptake in straw, roots and grains

Treatments		Carbon content (%)						Nitrogen content (%)						Nitrogen uptake (g per pot)					
		Straw		Roots		Grains		Straw		Roots		Grains		Straw		Roots		Grains	
			CD		SEd		CD		SEd		CD		SEd		CD		SEd		CD
C_{amb}	N_0	35.2		38.6		42.7		0.79		0.53		1.26		0.32		0.03		0.26	
	N_{150}	35.1		38.6		42.9		0.93		0.60		1.38		0.55		0.05		0.42	
	N_{200}	35.2		38.7		42.9		0.96		0.61		1.42		0.62		0.05		0.49	
C_{550}	N_0	39.4		39.5		42.8		0.70		0.48		1.09		0.30		0.04		0.24	
	N_{150}	39.5		39.7		42.9		0.90		0.54		1.32		0.59		0.06		0.53	
	N_{200}	39.5		39.7		43.2		0.92		0.59		1.44		0.64		0.07		0.64	
C_{750}	N_0	41.0		41.9		42.9		0.63		0.44		1.03		0.28		0.03		0.24	
	N_{150}	41.1		41.9		42.7		0.87		0.52		1.36		0.60		0.06		0.62	
	N_{200}	41.0		41.8		42.9		0.89		0.56		1.43		0.67		0.08		0.74	
		SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD	SEd	CD
C		0.22	0.45**	0.23	0.47**	0.25	NS	0.005	0.009**	0.003	0.006**	0.007	0.015**	0.003	0.006**	0.0003	0.0007**	0.003	0.006**
N		0.22	NS	0.23	NS	0.25	NS	0.005	0.009**	0.003	0.006**	0.007	0.015**	0.003	0.006**	0.0003	0.0007**	0.003	0.006**
C x N		0.38	NS	0.40	NS	0.43	NS	0.008	0.017**	0.005	0.011*	0.013	0.027**	0.005	0.010**	0.0006	0.0010**	0.005	0.010**

CO_2 levels : C_{amb} - 398 µ mol mol^{-1} CO_2; C_{550} - 550 µ mol mol^{-1} CO_2; C_2 - 750 µ mol mol^{-1} CO_2

Nitrogen levels : N_0 – 0 kg N ha^{-1}; N_{150} – 150 kg N ha^{-1}; N_{200} – 200 kg N ha^{-1}; * $P \leq 0.05$, ** $P \leq 0.01$, NS - Non significant

conformity with Uprety *et al.* (2000) who reported 10 to 15 % increase in the Carbon content of rice leaves, stem and roots exposed to elevated CO_2 of 575 to 620 µmol mol^{-1}over the ambient CO_2 of 370 µmol mol^{-1}. Thus, the combined effect of increased total biomass and Carbon content under elevated levels of CO_2 contributed for enhanced plant biomass Carbon stock to the tune of 22.5 – 34.4 %, 23.5 – 45.9 % and 24.5 – 39.8 %, respectively through straw, roots and grains over the ambient CO_2 (Fig. 3). On the other hand, elevated CO_2 significantly reduced the Nitrogen content of rice straw, roots and grains by 5.6 – 10.1 %, 6.9 – 12.1 % and 5.2 – 5.9 %, respectively over the current CO_2 concentrations. Our results are in accordance with (Weerakoon *et al.,* 2005; Seneweera *et al.,* 2005). This is further supported by Kim *et al.,* (2011) who reported that the elevated CO_2 of 622 µmol mol^{-1} reduced the nitrogen content of rice shoot, roots and grains by 15.8 %, 16.9 % and 21.0 %, respectively over the current CO_2 concentrations of 398 µmol mol^{-1}. It is obvious that these changes in carbon and nitrogen contents led to an increase in the C:N ratio of rice plants grown under elevated CO_2. The results of this study reveal that under elevated CO_2 the C:N ratio of rice roots, straw and grains were 82:1, 51:1 and 33.7 whereas the plants under ambient CO_2 recorded the C:N ratio of 66:1, 39:1, and 31:7 respectively. These observations are in conformity with the previous reports where (Uprety *et al*., 2000; Weerakoon *et al.,* 2005; Seneweera *et al.,* 2005; Gai-ping *et al.*, 2006) CO_2 enrichment increased rice plant's C:N ratio.

Overall, the results demonstrated that elevated CO_2 significantly increased the rice grain yield by 24.0 – 40.1 % over the current atmospheric CO_2 (Fig. 4). Similar findings have been observed by Kimball *et al.,* (2002) and Kim *et al., (*2011) which reported positive yield responses (5 to 71 %) to elevated CO_2 depending upon other factors such as temperature, cultivar and nutrition. Increased grain yield is evident from the fact that elevated CO_2 enhanced the accumulation of assimilation and the translocation from source (leaf or stem) to sink (panicle) (Sasaki *et al*., 2005; Fan *et al*., 2010). This study clearly demonstrated that the Nitrogen fertilization is a crucial factor for preventing down regulation of photosynthesis and stimulating the grain yield under elevated CO_2 levels. Meta-analysis study (Ainsworth, 2008) pointed out that low N treatments negated any enhancement in rice yield at elevated CO_2. Low nitrogen fertilization limited nitrogen uptake during vegetative growth, which constrained any increase in spikelet number, thereby limiting the yield response (Kim *et al.,* 2003). Further, low nitrogen may also cause more pronounced acclimation of photosynthesis to elevated CO_2, which can limit total dry matter (Suter *et al*., 2001; Ainsworth *et al*., 2003; Razzaque *et al*., 2011).

Our results demonstrated that Nitrogen fertilization beyond the current recommended dose is pivotal under rising CO_2 concentrations for managing the grain quality in terms of Nitrogen concentration. Sasaki *et al*., *(*2005); Kim *et al*., (2011); Wang *et al.,* (2011) who reported that elevated CO_2 significantly decreased Nitrogen or Protein concentration in rice grains. This suggests that when adequate nitrogen was not supplied, rice plants grown at high CO_2 became inferior to plants grown at ambient CO_2 in terms of grain quality. Though our results reported increased nitrogen uptake under elevated CO_2, grain nitrogen concentration decreased in the N_0 and N_{150} treatments. On the other hand, the increased grain nitrogen concentration in N_{200} suggest that the additional amount of nitrogen added might favoured enhanced nitrogen partitioning from source to sink. Our findings are in conformity with Weerakoon et al. (2005) who reported that rice grain nitrogen concentration increased at highest dose of Nitrogen as a result of improved nitrogen partitioning. Thus, CO_2 enrichment will favourably improve the productivity and nutritional quality only when the rice plants are nourished with excessive amounts of nitrogen.

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Add-on Information

Authors' contribution: S.K. Rajkishore: Planning and execution of Research work; **P. Doraisamy:** Mentor; **M. Maheswari:** Facilitation and guidance for lab experiments; **K.S. Subramanian:** OTC facility and methodology for nitrogen assimilatory assays; **R. Prabhu:** Graphical interpretation and editing; **G. Vanitha:** Statistical analysis.

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Influence of abiotic factors on stem borer incidence and species distribution in rice (*Oryza sativa* L.)

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Abstract

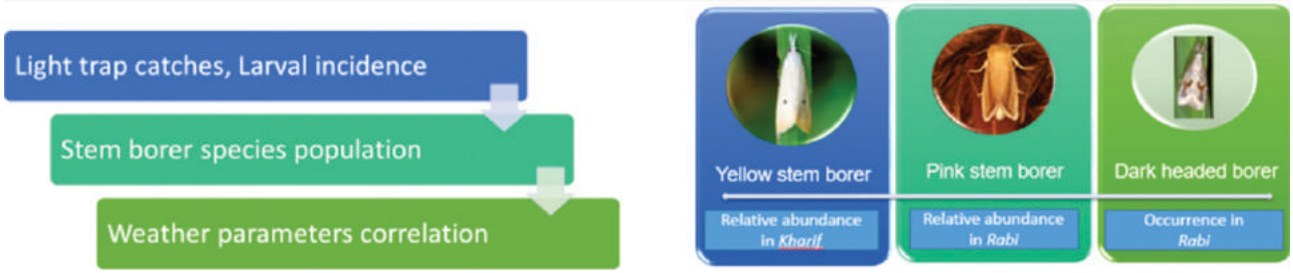
Aim: To evaluate the effect of abiotic factors such as temperature, relative humidity, wind velocity, sunshine hours and rainfall on the distribution and relative abundance of stem borer species in rice ecosystem.

Methodology: Seasonal incidence of stem borer species was monitored using light trap catches and were correlated with the weather parameters. The relative abundance of stem borer species during *Kharif* and *Rabi* was estimated based on light trap catches and larval incidence.

Results: Seasonal incidence revealed the occurrence of three stem borer species (yellow stem borer, pink stem borer and dark headed borer) populations in rice ecosystem. Yellow stem borer was found to dominate in rice ecosystem during *Kharif,* (80.61%) whereas during *Rabi,* pinkstem borer was found to be dominant (72.60%) than yellow stem borer (21.92%) and dark headed borer (5.48%). Weather parameters such as temperature, rainfall and wind velocity negatively influenced the occurrence of stem borer species in rice, whereas relative humidity and sunshine hours had positive effect. Regression analysis revealed the increased effect of abiotic factors such as temperature, relative humidity and wind velocityon the population of pink stem borer against yellow stem borer and dark headed borer.

Interpretation: The present study reveals that, the change in weather parameter during seasons, such as temperature (25 - 27°C), relative humidity (69 - 80 %) and wind velocity (3 - 4 km hr^{-1}) increased the fitness of pink stem borer species population than yellow stem borer and dark headed borer species in rice ecosystem, which was evidenced by the occurrence and relative abundance of pink stem borer population during *Kharif* 2018 and *Rabi* 2019 respectively.

Key words: Abiotic factors, Light trap catches, Rice, Stem borer



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Introduction

Rice (*Oryza sativa* L.) belonging to family Poaceae is an important grain crop in the world feeding more than 50 % of the human population (Fukagawa and Ziska, 2019). Globally, it is the second most cultivated cereal crop next to wheat. India ranks first in area (43.79 million ha) and second in rice production (109.70 MT) (Anon., 2018). Tamil Nadu is one among the major rice producing states in India. The productivity of rice crop is influenced by several biotic and abiotic factors. The rice crop is subjected to considerable damage by nearly 300 species of insect pests, among them 23 species are serious pests of rice (Pasalu *et al.,* 2006). Yield loss due to insect pests of rice have been estimated to be 25 % (Dhaliwal *et al.,* 2010). About 40% damage is caused by stem borer alone (Kumar *et al.,* 2019). The young larva of stem borer primarily enters into the leaf sheath and feeds for two to three days, after which the larva enters the basal part usually 5 to 10 cm above water level and feeds inside the stem causing dying of central shoot known as dead heart at vegetative stage and white ear head is formed by boring at the peduncle node during heading stage (Gupta *et al*., 2006). The yellow stem borer, *Scirpophaga incertulas* (Walker), the pink stem borer, *Sesamia inferens* (Walker) and dark headed borer *Chilo polychrysus* (Meyrick) are poplyphagous pests of the Gramineae. Various abiotic factors influence the occurrence and population dynamics of paddy stem borers (Gagan *et al.,* 2009). The changing climatic scenario with modern cultivation practices in rice crop has made pink stem borer to achieve key pest status in many rice growing regions of India, that causes dead heart, white ear and results in yield reduction (Sampath *et al.*, 2014). In view of the above, the present study was conducted to examine the influence of abiotic factors on the occurrence and relative dominance of stem borer species in rice ecosystem at Tiruchirappalli, Tamil Nadu.

Materials and Methods

Rice crop (cv. TRY 3) was grown at the experimental farm of Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirappalli District, Tamil Nadu, India, to monitor the seasonal incidence of rice stem borer species during *Kharif,* 2018 and *Rabi,*2019. Standard agronomic practices recommended by Tamil Nadu Agricultural University were adopted, except for plant protection measures and the rice crop was maintained during the entire period of study. A light trap unit made of galvanised iron sheet with a trapping device and collecting chamber was installed in the bund, at the centre of experimental area and operated from 7.00 p.m. to 11.00 p.m. with mercury vapour lamp of 160 W as the light source. The collecting jar with insecticide was changed every day and insects collected were counted each day and the species were assessed and sexed based on morphological characters (Hattori, 1971; Khan *et al.,* 1991; Sharma *et al.,* 2017) to arrive at weekly mean population.

The tillers expressing dead heart and white ear were cut opened at weekly intervals, during *Kharif,* 2018 and *Rabi,* 2019. The larvae collected were sorted out according to the morphological characters (Bhatt *et al.,* 2018) and occurrence of stem borer species was assessed.

The relative abundance of the stem borer species based on light trap catches and larval incidence during *Kharif* and *Rabi* was assessed by the following formula:

Relative abundance (%) = (Total no. of individuals of each species/ Total no. of individuals of all species) X100

Data on weather parameters such as maximum temperature (°C), minimum temperature (°C), relative humidity (%), wind velocity (km/hr), sunshine hours (hr) and rainfall (mm) were obtained from the Agro-meteorological Station at Anbil Dharmalingam Agricultural College and Research Institute, Tiruchirappalli. The weather parameters were correlated with the weekly light trap catches and their effect on stem borer species occurrence and distribution in the paddy ecosystem was estimated by correlation and regression analysis.

Results and Discussion

Based on light trap catches, adult populations of three stem borer species, such as Yellow stem borer (YSB), Pink stem borer and Dark headed borer (DHB) have been observed to occur in the rice ecosystem at Agricultural College and Research Institute, Tiruchirappalli. It is in accordance with the previous report of occurrence of Yellow stem borer, Pink stem borer and Dark headed borer stem borer species in major rice growing areas of Tamil Nadu (Ragini *et al*.2000).Periodical observations on the stem borer species moth population revealed the incidence of Yellow stem borer in the second week of August,32nd Standard Meteorological Week (SMW), Pink stem borer in the second week of September (38th SMW)and Dark headed borer in the third week of November (47th SMW) and also revealed the synchronous emergence of both the sexes (male and female adult moths) in all the three stem borer species observed. Baskaran *et al.* (2017) reported the appearance of first Yellow stem borer moth in light trap during the first week of August (31st SMW) which favours the present result. In the entire cropping season, the moth populations of Yellow stem borer reached its peak during the last week of December (52nd SMW) with males (13.38 no./week), females (30.88 no./week). The moth population of Pink stem borer reached its peak during the last week of December (52nd SMW) with males (36.86 no./week), females (31.86 no./week). The populations of Dark headed borer reached its peak during December (51st SMW) with trap catches of males (0.29 no./week) and females (1.71 no./week) during *Kharif* and *Rabi* (Fig. 1; Fig. 5). However, during *Kharif* 2018, the population of Yellow stem borer reached its peak during second week of October, 41st SMW and the population of PSB during fourth week of October, 43rd SMW. The present results are in accordance with the previous report that, the infestation of *S. incertulas* was found during August to September and December to January at Thirupathisaram, Kanyakumari, Tamil Nadu (Justin and Preetha, 2013).

Influence of weather parameters

The average temperature (30.82, 28.71 and 8.75), relative humidity (53.93, 60.00 and 71.93), wind velocity (15.91, 6.40 and

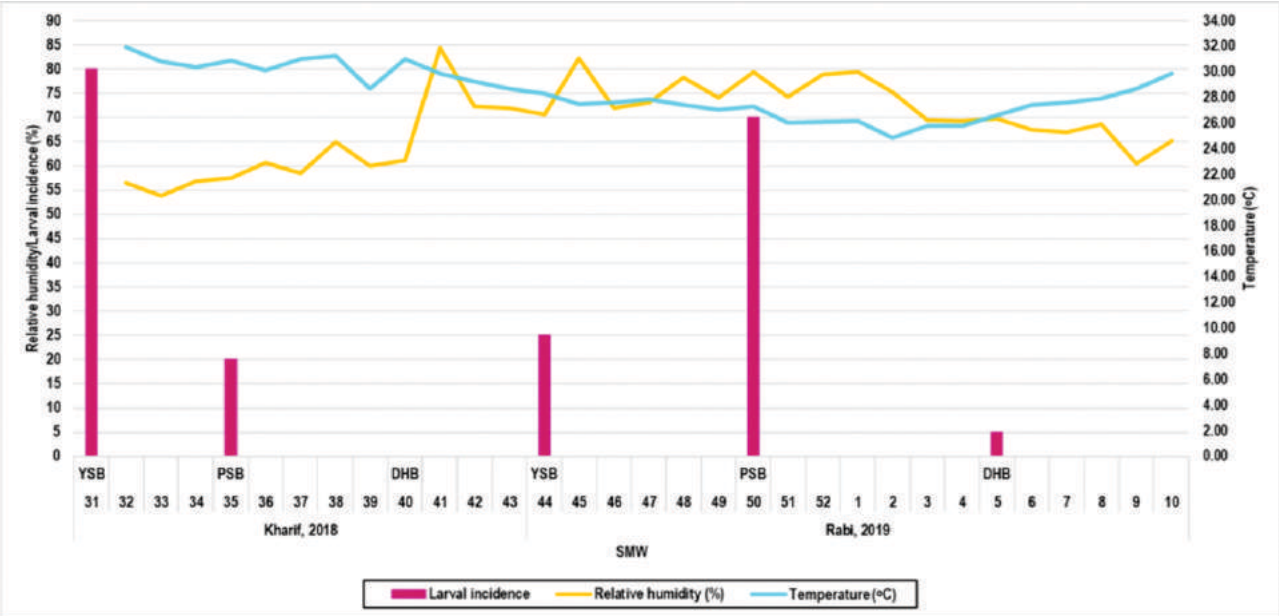

Fig. 1: Weather parameters temperature (°C) and relative humidity (%) observed during study.

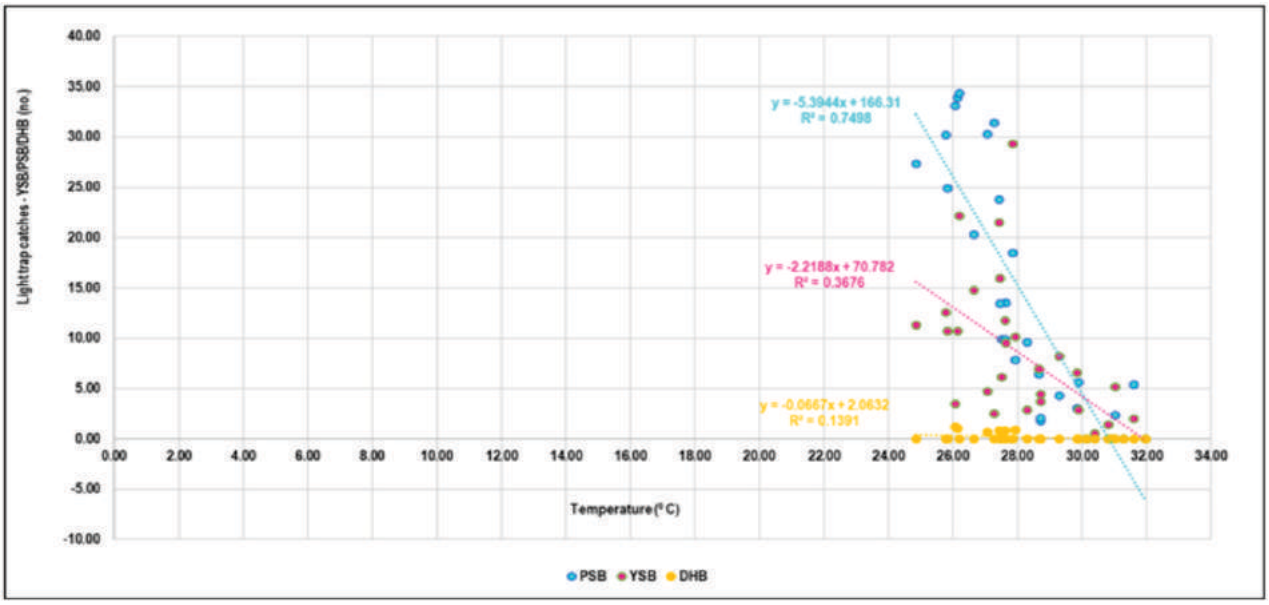

Fig. 2: Effect of temperature on the distribution of Yellow stem borer, Pink stem borer and Dark headed borer in rice ecosystem (*Kharif* 2018 and *Rabi* 2019).

3.03), sunshine hours (2.60, 6,59 and 3.83), and rainfall (0.00, 0.23 and 0.00) observed during 32nd, 38th and 42nd SMW favoured the incidence of adult population of YSB, PSB and DHB respectively (Fig. 1 and 5). Whereas, the weather parameters such as temperature (26.19 and 26.14), relative humidity (79.50 and 78.86), wind velocity (3.73 and 5.09) and sunshine hours (5.03 and 4.59) in these ranges observed during 52nd and 51st SMW respectively, favored the population of Yellow stem borer, Pink stem borer and Dark headed borer to reach their peak.

The results revealed that the occurrence of Pink stem borer required comparatively lower temperature, higher relative humidity, lower wind velocity, higher sunshine hours and higher rainfall than YSB during *Kharif* and *Rabi* season. Akhter *et al.* (2015) reported that, Pink stem borer required comparatively lower temperature for emergence than Yellow stem borer, *i.e.,* Pink stem borer was trapped below 32^{0}C and had strong relationship with temperature. Also, maximum moth catch of PSB was observed at 58-60% relative humidity, which is in line with the

Table 1: Influence of weather parameters on the light trap catches of rice stem borer species (*Kharif,* 2018 and *Rabi,* 2019)

Weather parameters	Stem borer species	Correlation co-efficient
Maximum temperature (°C)	YSB (M)	-0.515*
	YSB (F)	-0.581*
	PSB (M)	-0.801*
	PSB (F)	-0.821*
	DHB (M)	-0.352*
	DHB (F)	-0.387*
Minimum temperature (°C)	YSB (M)	-0.207
	YSB (F)	-0.679*
	PSB (M)	-0.725*
	PSB (F)	-0.830*
	DHB (M)	-0.243
	DHB (F)	-0.276
Relative humidity (%)	YSB (M)	0.452*
	YSB (F)	0.471*
	PSB (M)	0.988*
	PSB (F)	0.955*
	DHB (M)	0.234*
	DHB (F)	0.322*
Wind velocity (km hr^{-1})	YSB (M)	-0.398*
	YSB (F)	-0.407*
	PSB (M)	-0.521*
	PSB (F)	-0.553*
	DHB (M)	-0.200
	DHB (F)	-0.188
Sunshine (h per day)	YSB (M)	-0.106
	YSB (F)	0.070
	PSB (M)	0.140
	PSB (F)	0.208
	DHB (M)	0.163
	DHB (F)	0.011
Rainfall (mm)	YSB (M)	-0.391*
	YSB (F)	-0.027
	PSB (M)	-0.224
	PSB (F)	-0.263
	DHB (M)	-0.189
	DHB (F)	-0.187

M – Male, F- Female, *Significant at p=0.05

present findings. Maximum PSB adults were trapped, when the maximum temperature (29.7 and 36.4), minimum temperature (16.4 and 19.4) and relative humidity (67.0 and 47.5%) were observed in these ranges during the year 2013-14 and 2014-15 respectively (Deole, 2016).

It was clearly evident from the results, that the light trap catches of YSB male (r = -0.515*), YSB female (r = -0.581*), PSB female (r = -0.263*), PSB male (r= -0.224*), DHB male (r = -0.352*), and DHB female (r = -0.387*) showed a significant negative correlation with the maximum temperature (Table 1). The moth population also showed a significant negative correlation with the minimum temperature, YSB female (r = -0.679*), PSB female (r = -0.821*), PSB male (r= -0.189*). Relative humidity played a significant positive role on YSB male (r = 0.988*), YSB female (r = 0.471*), PSB male (r = 0.988*), PSB female (r = 0.955*), DHB male (r=0.234*) and DHB female (r=0.322*) population in the light trap catches. YSB male (r = -0.398*), YSB female (r = -0.407*), PSB male (r = -0.521*) and PSB female (r = -0.553*) showed a significant negative correlation with wind velocity. The sunshine hours was positively correlated with the moth population and did not have any significant effect on the occurrence of stem borers. The Rainfall had a negative correlation with the moth population, YSB male (r=-0.391*), YSB female (r=-0.027), PSB male (r=-0.244), PSB female (r=-0.263),DHB male (r =-0.189) and DHB female (r=-0.187) with a significant effect on YSB population (Table 1). Thus, the moth catch showed a significant negative correlation with the maximum and minimum temperature, rainfall and wind velocity, which indicated a decline in the population of stem borer species with increase in those abiotic factors. Whereas, relative humidity and sunshine hours had positive correlation with the moth catch of stem borer species. The population of stem borer species were found to increase as relative humidity and sunshine hours increased. Kakde and Patel (2014), documented that, the YSB population was positively correlated with relative humidity. Hugar *et al.* (2009) reported a significant positive correlation between YSB and sunshine hours and negative correlation with maximum temperature, which agrees

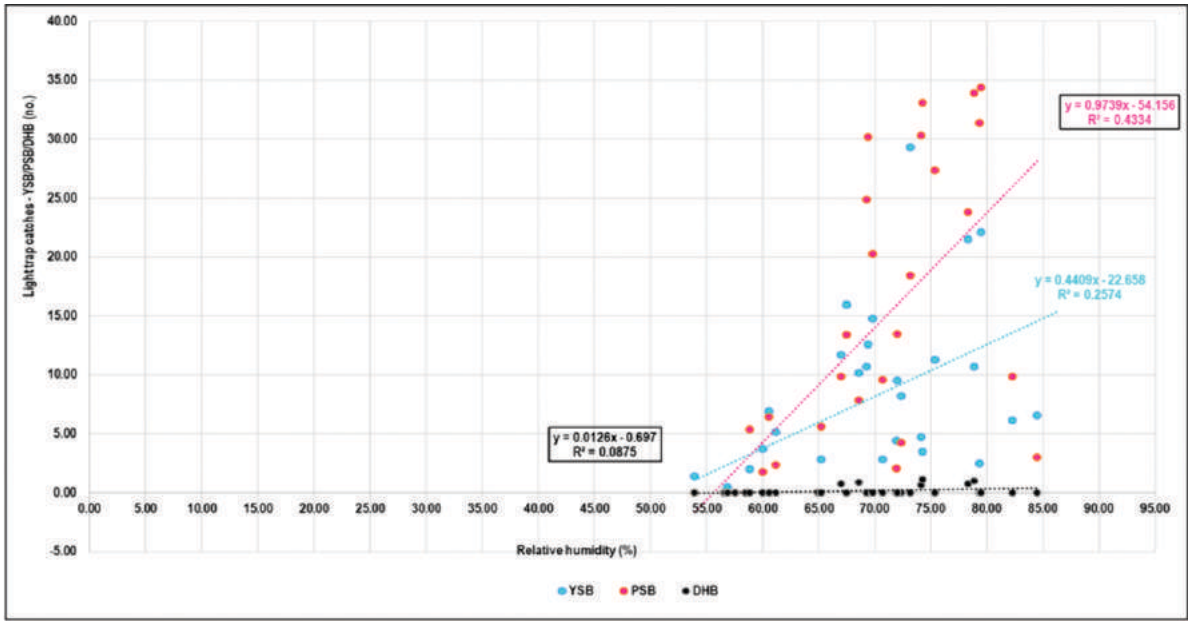

Fig. 3: Effect of relative humidity on the distribution of Yellow stem borer, Pink stem borer and Dark headed borer in rice ecosystem (*Kharif* 2018 and *Rabi* 2019).

with the present findings. Relative humidity was positively correlated with the trap catches by providing conducive environment for the emergence of adults of stem borer species, while rainfall was negatively correlated (Baskaran *et al*., 2017). Singh and Kular, (2015) reported the negative effect of maximum and minimum temperature and postivie effect of relative humidity on the population build up of PSB.

Thus the results on correlation between abiotic factors and stem borer species distribution based on light trap catches indicated that the seasonal distribution of stem borer species in rice ecosystem was significantly influenced by temperature, relative humidity and wind velocity. The linear regression equations indicated that in every one unit increase in temperature, 0.37%, 0.75% and 0.14% decrease in the

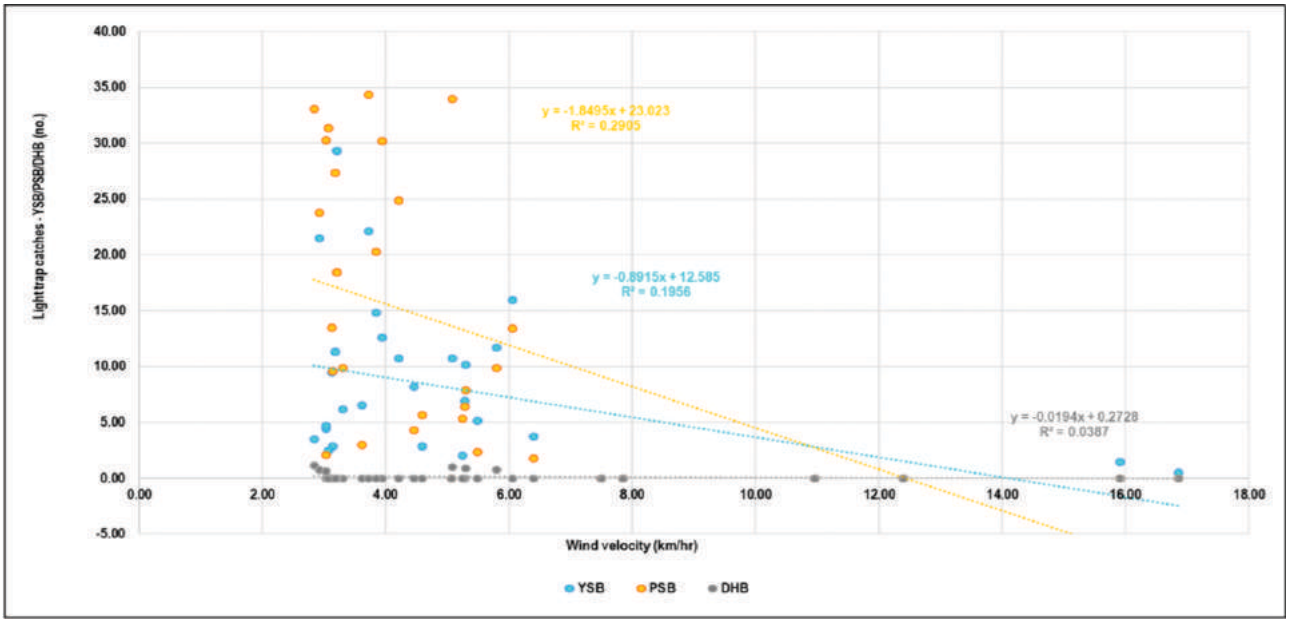

Fig. 4: Effect of wind velocity on the distribution of YSB, PSB and DHB in rice ecosystem (*Kharif*, 2018 and *Rabi*, 2019).

Table 2: Relative abundance of stem borer species in rice

Season	Relative abundance (%)*					
	Light trap catches			(Dead heart/ white ear) Larval incidence		
	Yellow stem borer	Pink stem borer	Dark headed borer	Yellow stem borer	Pink stem borer	Dark headed borer
***Kharif*, 2018**	80.61	19.39	0.00	80.00	20.00	0.00
***Rabi*, 2019**	21.92	72.60	5.48	19.12	75.00	5.88

*Mean of weakly observations

occurrence of YSB, PSB and DHB populations was found, in the rice ecosystem (Fig. 2). However, every one unit increase in relative humidity, there would be 0.26 %, 0.43%and 0.09 %increase in the incidence of YSB, PSB and DHB adult population respectively (Fig. 3). Similarly, each unit increase in wind velocity had 0.19, 0.29 and 0.04% effect in decreasing the populations of YSB, PSB and DHB respectively (Fig. 4). Adiroubane and Raja, (2010) reported that for every one unit increase in morning relative humidity, evening relative humidity, wind velocity, sunshine hours and dewfall, there would be 0.09, 0.08, 0.34, 0.09, 0.12 and 0.40 % increase of stem borer damage, respectively.

The relative abundance of stem borer species based on light trap catches showed the occurrence of YSB (80.61%), PSB (19.39%) and DHB (0.0%) during *Kharif,* 2018 and in *Rabi* 2019, witnessed PSB (72.60%), YSB (21.92%) and DHB (5.48%) (Table 2). Thus it clearly indicated the dominance of YSB during *Kharif,* 2018 and PSB during *Rabi,* 2019, which coincided with the maximum larval incidence of YSB (80.00%) during *Kharif* and PSB (75.00%)during *Rabi* (Fig. 1).This was in agreement with the findings that, *S. inferens* started egg laying in the field during 45th SMW to 48th SMW (first to last week of November) (Sharma *et al*., 2017). Baskaran *et al.* (2017) reported a reduction in the damage and trap collection of YSB, beyond 43rd SMW (*Kharif*), which coincides with the dominant occurrence of PSB during *Rabi* than the population of YSB in the present study. However, Gagan *et al*. (2009) reported the predominant occurrence of YSB throughout the season, while PSB was dominant towards the maturity of crop.

The present findings are further supported by the report that YSB was the most predominant species in both *Kar* (60.00%) and *Pishanam* (48.83%) season. PSB was found to be the second most dominating species in *Kar* (35.21%) and was as abundant as YSB in *Pishanam* (48.43%). DHB was found to have least occurrence in both the seasons (4.29-7.18%) (Ragini *et al*. 2000). The relative abundance of stem borer species and their natural enemies revealed that, YSB was dominant followed by DHB, PSB, white stem borer and striped stem borer (SSB) (Rahaman *et al*., 2014). Akhter *et al.* (2015) reported that, YSB and WSB were dominant from mid- March to May and from August to mid-October, while PSB was dominant from September to first week of April which is in line with the present findings of PSB in *Rabi.*

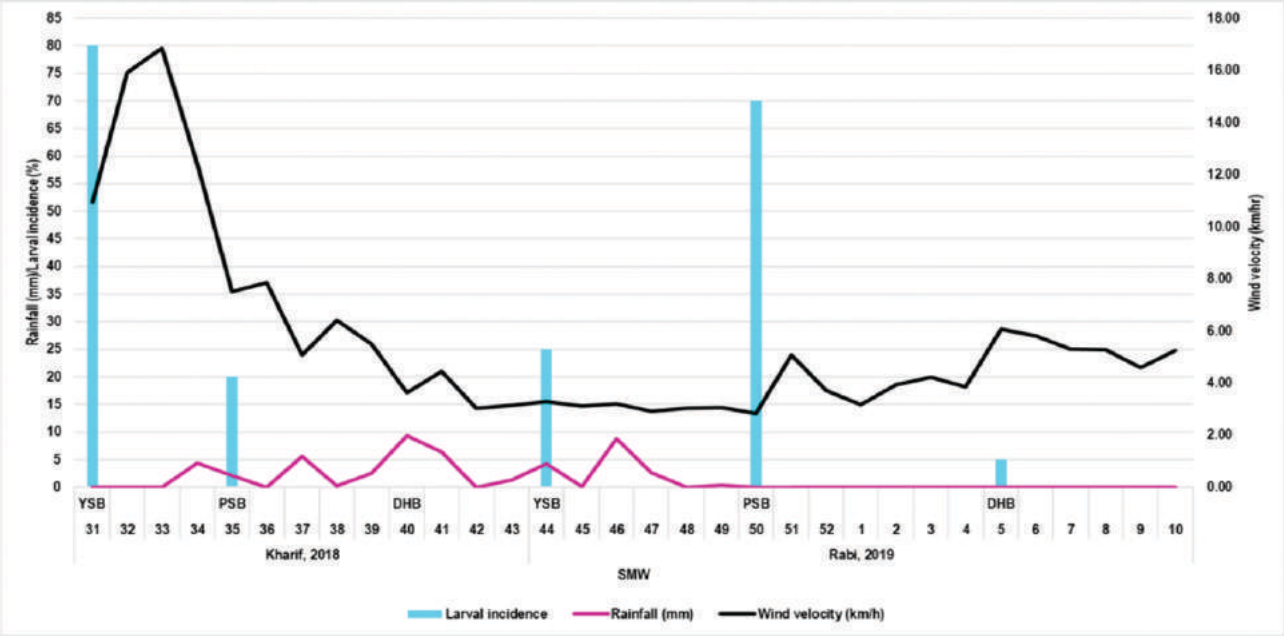

Fig. 5: Weather parameters rainfall (mm) and wind velocity (km hr^{-1}) observed during *Kharif* and *Rabi.*

The reduction in temperature and increase in relative humidity during *Rabi,* 2019 to *Kharif,* 2018 favoured the dominant occurrence of PSB than the YSB and DHB species during *Rabi* (Fig. 2 and 3). The data subjected to regression analysis confirmed the increased effect of temperature (R^2 = 0.75%), relative humidity (R^2 = 0.43%) and wind velocity (R^2 = 0.29%) on the population of PSB than YSB in rice ecosystem during *Kharif* and *Rabi.* Temperature (25 to 27°C), relative humidity (69 - 80%) and wind velocity (3-4 km hr^{-1}) increased the fitness of PSB than YSB and DHB species in rice ecosystem (Fig. 2, 3, 4). These factors favoured the dominant occurrence of PSB during *Rabi,* 2019 than YSB and DHB species.

Considering rice stem borer, YSB, PSB, DHB, SSB and WSB have been reported to occur in South - East Asia (Rahaman *et al.,* 2014). Among these, YSB is the widely distributed and predominantly occurring species than other stem borer species in rice ecosystem. YSB was more abundant (63.9%) than PSB (22.8%) and DHB (13.3%) in seven agro climatic regions of Tamil Nadu (Ragini *et al.* 2000). It is the fluctuations in the weather parameters, as climate change in the long run influenced the incidence and relative abundance of PSB species in the rice ecosystem.

Jiang *et al.* (2005) reported that increase in each unit of white ear damage has a greater impact on rice yield. Globally, 50 % of the insecticides are used to manage the pest menace in rice crop (Huesing and English, 2004). Many conventional insecticides though have been evaluated against rice stem borer, yet they have failed to provide the adequate control. It is due to variation in the distribution and relative abundance of stem borer species, which was strongly influenced by the fluctuations in abiotic factors. Pesticides which were effective in managing a stem borer species showed reduced effect in managing other stem borer species population. It can be well supported by the findings of Visagie (2016) that among 14 insecticides evaluated for controlling three stem borer species in maize, two insecticides, chlorantraniliprole and chlorfenapyr were less effective in controlling *B. fusca*, and were effective in controlling *C. partellus and Sesamia calamistis* Hampson.

Hence, before developing the management tactics for stem borer in rice ecosystem based on visual symptoms, the seasonal abundance of stem borer species has to be ascertained through light trap catches and larval incidence for the development of species specific control strategy. Also, weather based forecasting models on the occurrence and relative abundance of stem borer species in rice ecosystem can be developed for an effective management of rice stem borer, thereby increasing the production and food security.

Add-on Information

Authors' contribution: T. Sharmitha: Carried out research and drafted manuscript ; **C. Gailce Leo Justin:** Major advisor and contributor in conceiving the research problem, implementing and compilation of data; **S. Sheeba Joyce Roseleen:** Minor advisor, Co-contributor in interpretation of results.

Research content: The research content is original and has not been published elsewhere.

Ethical approval: Not Applicable

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not Applicable

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Estimation of carbon footprint in Tamil Nadu Agricultural University, Coimbatore

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Abstract

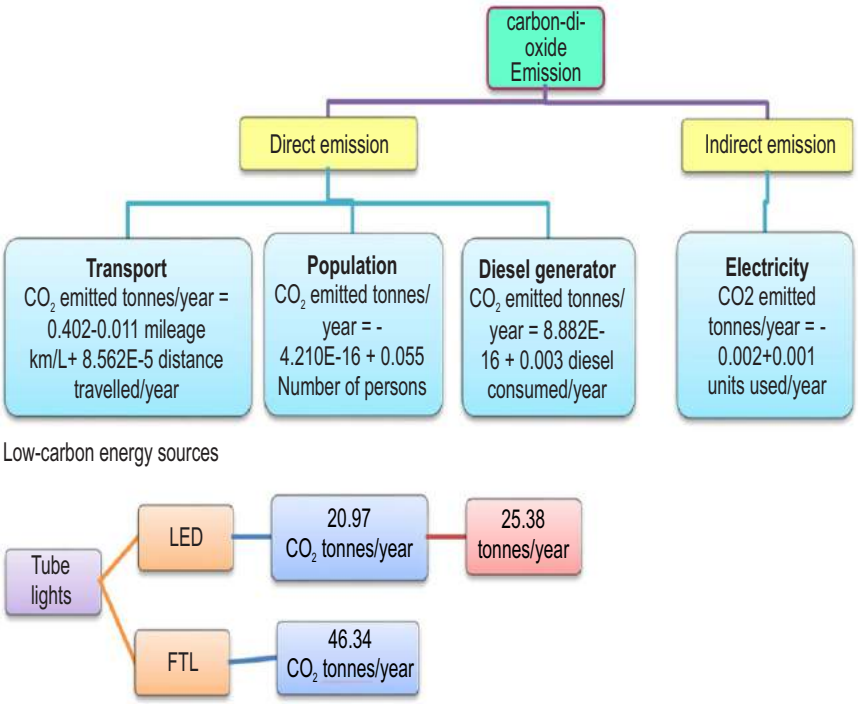
Aim: A study was conducted in the Eastern block of Tamil Nadu Agricultural University, Coimbatore to estimate the carbon footprint from various sources comprising of transport, human population, diesel generator and electricity use.

Methodology: The data were collected based on the questionnaire developed. Empirical equations were used for computation and data were statistically analysed.

Results: Carbon footprint analysis in the selected buildings revealed that, the equivalent carbon emission from power, diesel generators, human population and transport were 291, 14.17, 78.72 and 36.43 tonnes per year, respectively. It was inferred that emission from power use was greater in comparison with emission from transport, human population and dieselgenerators.

Interpretation: For a diversified source of emission in a university, reduction in anthropogenic emissions can be achieved by increasing the capacity of carbon sinks, *i.e.*, through carbon sequestration and switching to low-carbon energy sources.

Key words: Climate change, Carbon footprint, Carbon sink, Low-carbon energy sources



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Introduction

Most of the observed increase in global average temperature is due to steady increase of CO_2 in the atmosphere. Greenhouse gas emissions cause heat to be trapped by the earth's atmosphere, and this has been the main driving force behind global warming. The main sources of such emissions are natural systems and human activities. Yue and Gao, statistically analysed global greenhouse gas emissions from natural systems and anthropogenic activities and concluded that the earth's natural system can be considered as self balancing and that anthropogenic emissions add extra pressure to the earth system (Yue and Gao, 2018). Poverty, population pressure, agricultural expansion and intensification and development of infrastructure have been suggested as major threats to biodiversity in the tropics (Davidar *et al.,* 2010) which increases the pressure on the remaining forests (Bargali *et al.,* 1992a, b; 1993; Kittur *et al.,* 2014, Baboo *et al.,* 2017; Bargali *et al.,* 2018).

Continuous increase in the human population together with livestock populations, the pressure on these forests in terms of intensive livestock grazing, fuel wood and timber harvesting for their energy and income generation are mounting and consequently resulting in the reduced carrying capacity of these forests (Davidar *et al.,* 2010; Sagar and Singh 2004) which increased the change in local atmosphere (Arora *et al.,* 2011; Bargali *et al.,* 2019; Manral *et al.,* 2020). Even CH_4 and chlorofluorocarbons have significant importance but their concentration is significantly less than CO_2. Anthropogenic activity has been increasing the concentration of greenhouse gases in the atmosphere. Following the rule that only measurable is manageable, ministration of greenhouse gas intensiveness of different products, bodies, and processes is going on worldwide (Pandey, 2011). The Carbon footprint is made up of the sum of two parts, the direct and indirect or secondary footprint (Tukker and Jansen, 2006).

Electricity production generates the largest share of greenhouse gas emissions. Approximately, 67% of our electricity comes from burning fossil fuels, mostly coal and natural gas (USEIA, 2016). Transportation contributes 26% of greenhouse gas emission. Emissions from transportation primarily come from the exhaust of cars, trucks, ships, trains and planes. Over 90 % of the fuel used for transportation is petroleum based, which includes gasoline and diesel (Kahn Ribeiro, *et al.,* 2007). Letete,et al. (2011) had estimated carbon footprint for the year 2007 at University of Cape Town's to be about 83400 tons CO2 equivalent, with campus energy consumption, Transportation and Goods Services contributes about 81%, 18% and one% footprint. Electricity consumption alone contributes about 80% of all the emissions associated with university activities. Carbon Dioxide from various emission inventories in Institute of Science and Technology, Arakkunnam was carried out by Mary Lissy (2012) and suggested remedial measures for the reduction of emissions as a part of social commitment. Utaraskul, (2015) had studied the Carbon footprint of 35 students in Environmental Science Program, Faculty of Science and Technology, Suan Sunandha Rajabhat University based on three criteria transportation, food consumption and energy consumption by using electric appliances. Considering all these factors a study was conducted in the Eastern side of Tamil Nadu Agricultural University, Coimbatore to estimate the carbon footprint from various sources comprising of transport, human population, diesel generator and electricity use.

Materials and Methods

The study was carried out in the eastern block of Tamil Nadu Agricultural University, Coimbatore. The study area is 77606 sqm comprising of 29 buildings.

Data collection: The data were collected from 29 buildings based on the semi-structured questionnaire developed and interaction with the individuals (respondents).

Estimation of carbon footprint

Estimation of CO_2 dioxide emission from electrical appliances:

Total units consumed by electrical appliances (x) = b*c*d/1000 (kWh)..... (1)

Type of fitting (lights, fans etc.,) (a) Wattage of the fitting. (b), Installed fixtures in numbers. (c), Average working hours in a year. (d), CO_2 emission factor. (e)

Total CO_2 emission from electrical appliances = x*e..... (2)

Estimation of CO_2 emission from diesel generators:

Total CO_2 emission from generators=b*c..... (3)

Diesel consumed litres per year (b) CO_2 emission factor©

Estimation of CO_2 emission from human population:

Total CO_2 emission from human beings=a*b*c*d ..... (4)

Total number of individuals in the building. (a) Average working hours in a week; (b) Average working days in a year. (c) Per capita CO_2 emission

(0.9 kilograms of CO_2 for each day per person).

Estimation of vehicular CO_2 emission

Total CO_2 emission from vehicles=c*e.....(5)

Distance travelled. ©, CO_2 emission factor. (e)

[To be taken into consideration: type of vehicle (two wheeler, four wheeler etc.,), type of fuel (diesel or petrol) and mileage of vehicle].

Note: The emission factors for vehicles were assumed from the report given by "Automotive Research Association of India", 2007. When the emission factors were unavailable in the database, the constant emission factors for petrol and diesel were assumed. Total CO_2 emission = Electrical appliances carbon dioxide emission+ CO_2 emission from diesel generators + Human CO_2 emission +CO_2 emission from vehicles ..... (6)

Statistical analysis: The data obtained were statistically analysed to evaluate possible significance of independent variables to dependant variable.

Multiple linear regression model: A model which depicts the influence of two or more independent variables on the dependent variable. In this model, the dependent variable will be a continuous variable and independent variables can be continuous, discrete andcategorical.

Multiple linear regression model is represented by:

$$Y= a+b_1x_1 +b_2x_2+\ldots\ldots b_px_p$$

Where, a is the constant and b_1, b_2 etc. are the partial regression coefficients, x_1, x_2, x_p are the p independent variables. Partial regression coefficients show the influence of independent variable to dependent variable. This can be tested for its significance by applying Student's t-test, the coefficient of multiple determination R^2 is the contribution of all independent variables considered in the model to dependent variable.

Results and Discussion

The power use in buildings and the equivalent carbon emitted was computed using Eq.1 and 2, respectively. Fig. 1. Shows that the Carbon emission was highest in the department of Agricultural Engineering College and Research Institute (24.82 tonnes per year), followed by the crop physiology department (19.57 tonnes per year) and technology parks. Total power consumed 341865 kWh/year and the equivalent Carbon emission was 291 tonnes per year in the study area.

One of the options to reduce carbon emission is to replace the existing fluorescent tube lights in the buildings with LED which can bring about a considerable reduction in CO_2 emission as well as cost. Table 1 reveals that the carbon emission from FTL and LED was 46.342 and 20.966 tonnes per year respectively. It is evident that there is a difference in emission reduction of 25.376 tonnes of CO_2 per year. The unit saved by replacing FTL to LED is 29853.750 kWh per year. It is interpreted that out of 29 buildings only 10 buildings used diesel generator and the overall diesel consumption per year was 5340 l, with equivalent CO_2 emission of 14.17 tonnes per year. Fig.2 reveals that the maximum emission was found in Physical Sciences and Information Technology Department. The total population in the study area was 1428 and the CO_2 emitted from the population in different buildings was 78.72 tonnes per year. Fig. 3 represents the CO_2 emission plotted against population in the buildings. It is

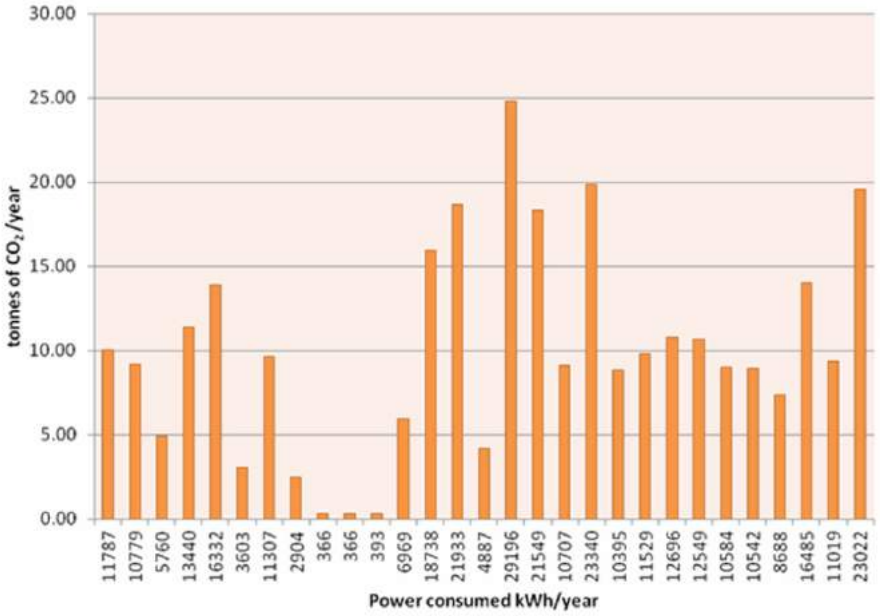

Fig.1: Equivalent carbon dioxide emission from power use.

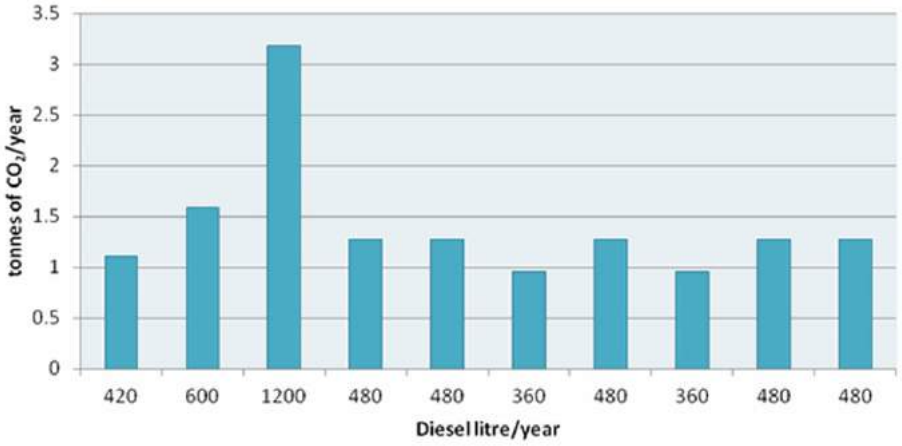

Fig. 2: Equivalent carbon dioxide emission from diesel generators.

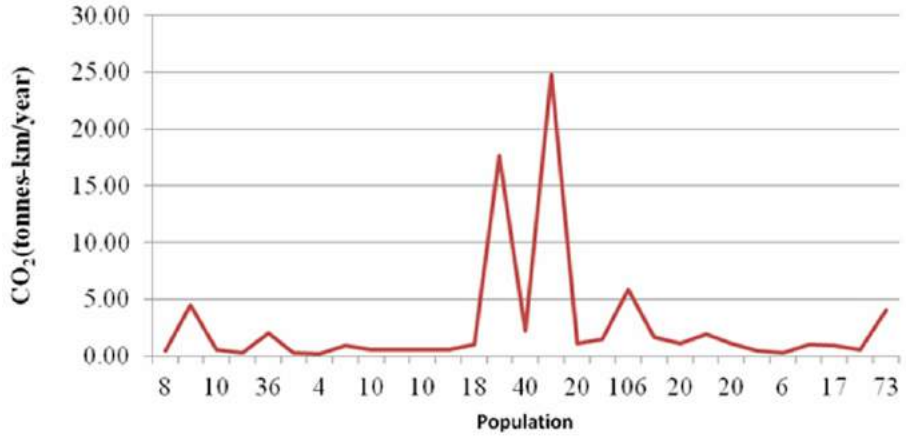

Fig. 3: Equivalent carbon dioxide emission from human population.

inferred that as the population increased the CO_2 emission also increased drastically and vice-versa.

Vehicles were categorized according to their type and the average values of petrol and diesel consumed, mileage, distance travelled and CO_2 emitted is presented in Fig. 4. The maximum CO_2 emission was observed in bus (1.671 tonne sper year) and the minimum for two wheelers between 110 to 200 CC (0.080 tonnes per year). The total Carbon emission from transport was estimated to be 36. 43 tonnes per year. The total contribution from different sectors to carbon emission is given in Fig. 5. It can be inferred that emission from power use was higher in comparison with emission from transport, human population and diesel generators. This is in accordance with the study carried out by Letete *et al.* (2011). Letete *et al.* (2011 who had estimated carbon footprint for the year 2007 at University of Cape Town's and inferred that Electricity consumption alone contributed about 80% of all the emissions associated with university activities. A multiple linear regression equation was fitted by considering CO_2 emitted as the dependant variable and electrical units consumed, human population, transport (mileage and distanced travelled) and diesel as independent variables and the results are presented in Table 2.

The mean units consumed, human population, mileage, distanced travelled, and diesel consumed was found to be 1180.000 (kWh per year), 47.600, 41.000 (km l^{-1}), 2625.980 (km per year), 534.000 (L per year) with the standard eviation values of 7361.908, 95.772, 16.279, 1725.069, 244.049 units respectively. Four regression equations were fitted by taking units, consumed, human population, transport (mileage and distanced travelled) and diesel as independent variables and CO_2 emitted as dependent variable. The results showed that for a unit increase in units consumed, CO_2 increased to 0.001 units and the following equation is CO_2 emitted tonnes per year = -0.002 +

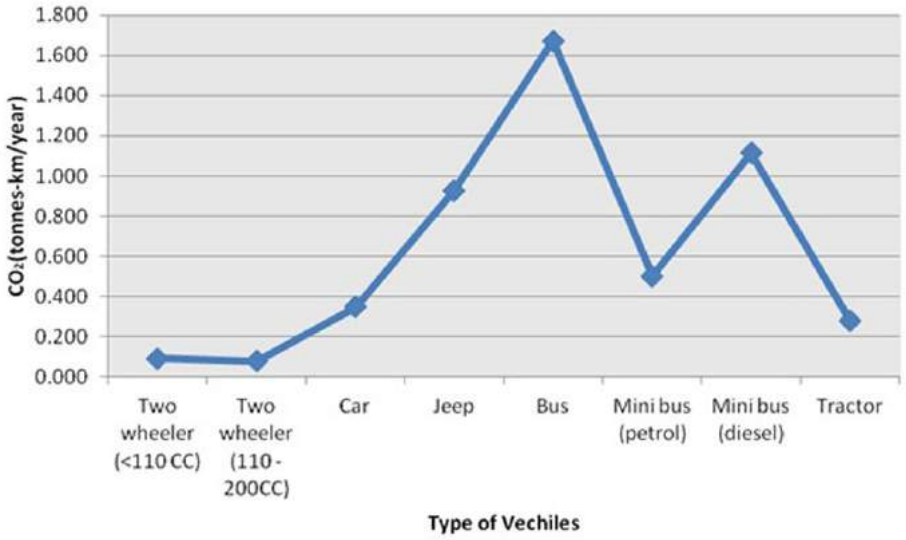

Fig. 4: Equivalent carbon dioxide emission from vehicular transport.

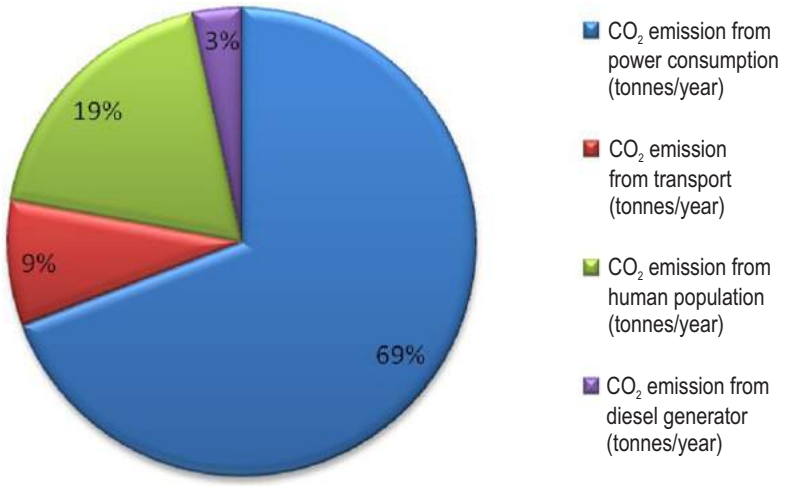

Fig. 5: Overall carbon dioxide emission from different sources.

0.001 units consumed per year For a unit increase in human population, CO_2 emission increased to 0.055 units CO_2 emitted tonnes per year = -8.210E-16 + 0.055 number of persons.

The results of regression analysis with mileage and distance travelled as independent variables showed that for a unit increase in the distance travelled the CO_2 emission increased to 8.562E-5 and for a unit increase in the mileage the CO_2 emission decreased to 0.011 units. CO_2 emitted tonnes per year =0.402-0.011 mileage km l^{-1} + 8.562E-5 distance travelled per year For a unit increase in the diesel consumed by diesel generator, the CO_2 emission increased to 0.3 units CO_2 emitted tonnes per year = 8.882E-16 + 0.003 diesel consumed.

It was inferred that emission from power use (69%) was greater in comparison with emission from transport (9%), human population (19%) and diesel generators (3%). Potential actions to mitigate emissions include increased energy conservation and efficiency, employment of renewable energy systems and use of alternative fuels. Other greenhouse gas mitigation options include sequestration of CO_2 in biologic sinks such as plant biomass. The problem of anthropogenic CO_2 accumulation in the atmosphere can be addressed either by reducing CO_2 emission or by developing carbon sinks.

Table 1: Comparison of fluorescent tube light and LED

Buildings	Electrical units used (kWh per year)		CO_2 emitted (tonnes per year)		Units saved (kWh per year)	CO_2 emission reduction (tonnes per year)
	FTL	LED	FTL	LED		
Mushroom Research Training Centre	1360.800	612.360	1.157	0.521	748.440	0.636
Agro Climate Research Center (ACRC) & Press	4859.400	2186.730	4.130	1.859	2672.670	2.272
Community Radio Station	1050.000	472.500	0.893	0.402	577.500	0.491
Directorate of Open and Distance Learning (ODL)	1890.000	850.500	1.607	0.723	1039.500	0.884
Agricultural Technology Information Center (ATIC)	1008.000	453.600	0.857	0.386	554.400	0.471
Sustainable Laboratory	252.000	113.400	0.214	0.096	138.600	0.118
Department of Sustainable Organic Agriculture	1965.600	982.800	1.671	0.835	982.800	0.835
Technology Park 2	134.400	60.480	0.114	0.051	73.920	0.063
Technology Park 3	134.400	60.490	0.114	0.051	73.910	0.063
Technology Park 4	134.400	60.500	0.114	0.051	73.900	0.063
Technology Park 6	134.400	60.510	0.114	0.051	73.890	0.063
Physical Sciences and Information Technology	6678.000	3005.100	5.676	2.554	3672.900	3.122
Agricultural Engineering College & Research Institute	9777.600	4399.920	8.311	3.740	5377.680	4.571
Water Technology Center (WTC)	1512.000	680.400	1.285	0.578	831.600	0.707
Nematology	1722.000	774.900	1.464	0.659	947.100	0.805
Pathology	6115.200	2751.840	5.198	2.339	3363.360	2.859
Directorate of Agri Business Development	3024.000	1360.800	2.570	1.157	1663.200	1.414
Technology Business Incubator	2419.200	1088.640	2.056	0.925	1330.560	1.131
Bioenergy Workshop	1932.000	869.400	1.642	0.739	1062.600	0.903
Food and Agricultural Pprocess Engineering Laboratory	1386.000	623.700	1.178	0.530	762.300	0.648
Farm Machinery Workshop	1764.000	793.800	1.499	0.675	970.200	0.825
Soil and Water Conservation Workshop	2469.600	1111.320	2.099	0.945	1358.280	1.155
Radio Isotope Laboratory	1201.200	540.540	1.021	0.459	660.660	0.562
Pesticide Toxicology Laboratory I	714.000	321.300	0.607	0.273	392.700	0.334
Pesticide toxicology laboratory II	201.600	90.720	0.171	0.077	110.880	0.094
Crop Physiology	680.400	340.200	0.578	0.289	340.200	0.289
Total	54520.200	24666.450	46.342	20.966	29853.750	25.376

Table 2: Regression analysis for overall carbon emission

Model	Electricity consumed kWh per year	Human Population/ day	Transport		Diesel generator
			Mileage km l^{-1}	Distance travelled (km per year)	
Mean	1180.000	47.600	41.000	2625.98	534.000
Standard deviation	7361.908	95.772	16.279	1725.069	244.049
Constant	0.002	8.210E-16	0.402		8.88E-16
Regression co-efficient values	0.001	0.055	0.011	8.56E-05	0.003

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Add-on Information

Authors' contribution: C.G. Karishma: Conceived and designed the analysis data collection, performed the analysis and wrote the paper; **S.V. Kottiswaran:** Technical guidance, supervised the research and contributed to final manuscript; **A. Balasubramanian:** Technical guidance, supervised the research and contributed to the final manuscript; **B. Kannan:** Technical guidance, supervised the research and contributed to the final manuscript.

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Butterfly diversity in relation to host and nectar food plants in TNAU Botanical Garden, Coimbatore

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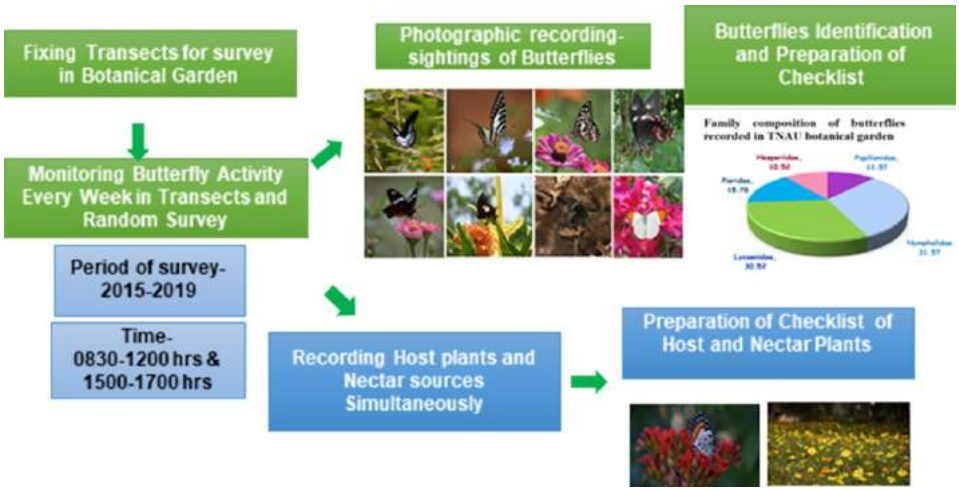
Abstract

Aim: The present study was undertaken to assess the food resources and butterfly diversity in Botanical Garden, Tamil Nadu Agricultural University.

Methodology: The survey was conducted in targeted locality from September 2015 – October 2019 and butterflies sightings were recorded using a digital camera (Nikon D7200 with kit lens 18-105 mm, Tamron 200-300 apo dg macro lens).



Results: Total 95 species of butterflies were recorded in the garden. Among all families, the highest count was observed in Nymphalidae family followed by Lycaenidae (29 species), Pieridae (15 species), Hesperiidae (10 species) and Papilionidae (11 species). The relative diversity of Nymphalidae (31.57 %) and Lycaenidae (30.52 %) were higher. For Pieridae, RD value accounted for 15.78 % and 11.57 % for Papilionidae. RD value was least for Hesperiidae family (10.52 %). Out of 95 butterflies, the host plants of 88 butterflies were present in the garden itself. Fifteen nectar plants belonging to eleven families were most preferred nectar sources.

Interpretation: The present study gives information of butterfly and its food plant diversity in the garden. The obtained information will help in planting additional host and nectar plant resources to attract rare species. It also sheds light on the importance of survival of prominent nectar plants throughout the year for maintaining the butterfly abundance in garden.

Key words: Butterfly, Conservation, Diversity, Host plants

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Introduction

Butterflies are scaly winged colourful insects belonging to the Order Lepidoptera and suborder Rhopalocera. Butterflies play a prominent role in pollination (Kunte 2000, Tiple *et al.,* 2006, Atmowidi *et al.,* 2007, Mukherjee *et al.,* 2015) and also serve as both prey and predator in the food chain (Hammond and Miller 1998, Rusman *et al.,* 2016). The birds plan their entire breeding season based on the abundance of caterpillars. Learning about biodiversity provides insight into the mechanism and also explains the valuable ecosystem services rendered by the biological elements to the human kind. Survey and monitoring of species diversity in a particular ecosystem help in understanding the valuable role played by the species. Considering this aspect with reference to butterflies, they serve as indicators of a healthy ecosystem (Hill 1999; Kocher and Williams 2000; Koh and Sodhi 2004; Thomas 2005; Koh 2007). They provide a deeper insight of spatio-temporal patterns of diversity both ecologically and taxonomically in urban frameworks (Bonebrake *et al.,* 2010; Samanta *et al.,* 2017).

These scaly winged Lepidopterans have intimate relationship with plants, and their life cycles are exceptionally interlinked. (Feltwell, 1986). Ehrlich and Raven (1964) explained that butterflies are herbivores coevolved with plants and they developed a model for co-evolution of plants and butterflies. The food requirements of adult butterflies and caterpillars differ; adults rely on nectar sources like flowers and ripe fruits whereas the caterpillars require specific host plants for meeting out their foliage needs (Nimbalkar *et al.,* 2011). Habitat fragmentation and decline in food resources including host and nectar plants are known to cause severe impact on butterfly populations (Clark *et al.,* 2007; Di Mauro *et al.,* 2007). Considering the ecosystem services rendered by the butterflies and to promote the conservation, the present study was taken to assess butterfly diversity by preparing a checklist of butterflies in the Botanical Garden of TNAU and identify the host and nectar resources.

Materials and Methods

The botanical garden is located in the premises of TNAU campus, around 3.5 km from Coimbatore city, Tamil Nadu. The garden lies between 11.0418ºN and 76.93ºE at an elevation of about 431 m. The botanical garden occupies an area of about 300 acres and it hosts a wide variety of flower crops, ornamental trees and succulents. Flower crops were raised periodically and their presence influenced the diversity of butterflies. Butterflies belonging to five families namely- Papilionidae, Nymphalidae, Hesperiidae, Pieridae and Lycaenidae were observed. Regular weekly visits were made for observing butterfly activity throughout the year following the method of Pollard (1991).

The study area was intensively explored for past five years (2015-2019) to assess the diversity, nectar food sources and conservation priorities of butterflies. Observations were done between 08.30 am. and 12.00 pm. hrs (morning) and between 15:00 and 17:00 hrs. During the survey, butterflies were classified into five categories based on their abundance. The sighting of butterflies found 80-100% time were categorized as very common, 60-80% as common, 40-60% as uncommon, 10 – 40% as rare and below 10% as very rare. Images were captured with Nikon D 7200 camera fitted with 18-105 mm lens and none of the live specimens were collected. Field guides and books were used for the identification of butterflies (Bhakare *et al.,* 2018; Evans,1927, Gunathilagaraj *et al.,* 2015; Kehimkar,2008; Wynter blyth,1957).

Early stage of butterflies including egg, larval and pupae were recorded. Identification of host plants and nectar plants were done based on the information in the butterfly field guides (Bhakare *et al.,* 2018; Kehimkar, 2008). The study area was intensively explored for the past five years (2015-2019) to assess the diversity, nectar food sources and conservation priorities of butterflies. Relative diversity was put forth as percentage of occurrence. It was calculated the following formula:

$$\text{Percentage Occurrence} = \frac{\text{No. of species observed in each family}}{\text{Total number of species observed}} * 100$$

The butterfly diversity was assessed using Shannon diversity index and Simpson's Index of diversity:

$$\text{Shannon diversity index } H' = -\sum pi \ln pi$$

Where, pi denotes proportion of individuals in species and is estimated using pi=ni/N: ni is the number of individuals in species and N is the total number of individuals in the community.

$$\text{Simpson's Index of diversity } D = 1 - \left(\frac{\Sigma n(n-1)}{N(N-1)}\right)$$

where, n is the total number of organisms of a particular species; N is the total number of organisms of all species.

Host plants present in the garden were recorded and are presented in Table. 1. Fifteen nectar plants belonging to family Acanthaceae, Amaranthaceae, Asteraceae, Malvaceae, Nyctaginaceae, Lamiaceae, Rubiaceae and Verbanaceae were recorded.

Results and Discussion

Total 95 species of butterflies were recorded in the garden. The details of recorded butterflies are furnished in Table 1. Family composition of butterflies are presented in Fig. 1. The species count of Nymphalidae was highest (30 species), followed by Lycaenidae (29 species), Pieridae (15 species), Hesperiidae (10 species) and Papilionidae (11 species). The relative diversity value was highest for family Nymphalidae (31.57%), followed by Lycaenidae (30.52%). For Pieridae, relative diversity value accounted for 15.78% and 11.57% for Papilionidae. It was least for Hesperiidae family (10.52%). Considering, Sub-family wise distribution (Fig. 2), among 17 subfamilies, the species count of Polyommatinae (18), Lycaenidae was highest followed by

Pierinae (12) of Pieridae and Papilioninae (11) of Papilionidae. The lowest species count of one was observed in the Charaxinae of Nymphalidae, two subfamilies of Lycaenidae-Miletinae and Curetinae and Coeliadinae of Hesperiidae. Diversity indices values were 4.14 based on Shannon diversity index and 0.98 based on Simpson's Diversity index.

These values obtained from dominance and Information Statistic Indices showed the abundance, rich butterfly diversity and evenness of species existing in the garden. Photographs of butterflies are presented in Fig. 1-5 representing five families. Early life stages of butterflies are presented in Fig. 8. The observed butterflies *Castalius rosimon, Deudorix isocrates, Deudorix epijarbas, Pachliopta hector* comes under Schedule I part IV of Indian Wildlife Protection Act, 1972. *Lampides boeticus, Charaxes solon, Euthalia aconthea, Hypolimnas missipus, Cepora nerissa* are placed in Schedule II part II of Indian Wildlife Protection act, 1972. *Euploea core* and *Appias libythea* are placed under Schedule IV.

The Botanical Garden hosts wide variety of nectar plants like *Aster sp.*, *Bougainvillea glabra*, *Celosia argentea, Cosmos sulphureus, Clerodendrum sp., Eupatorium odoratum, Gomphrena globosa, Ixora coccinea, Justicia adhatoda, Lantana camara, Morinda citrifolia, Solidago canadensis, Tagetes sp.* and *Zinnia elegans*. Apart from the cultivated plants, natural patches of weeds like *Tridax procumbens* serve as a prominent nectar source as they flower throughout the year. Similar finding was

Table 1: List of butterflies observed in the Botanical Garden, TNAU

Scientific Name	Common Name	Status	Host plants in Garden
Family: Papilionidae, Subfamily: Papilioninae			
Graphium doson (C.&R. Felder,1864)	Common Jay	C	*Polyalthia longifolia*
Graphium agamemnon (Linnaeus,1758)	Tailed Jay	C	*Polyalthia longifolia*
Graphium nomius (Esper,1785)	Spot Swordtail	C	*Polyalthia longifolia*
Papilio polytes (Linnaeus,1758)	Common Mormon	VC	*Citrus sp, Murraya paniculata*
Papilio helenus (Linnaeus,1758)	Red Helen	VR	*Citrus sp*
Papilio polymnestor (Cramer, 1779)	Blue Mormon	R	*Citrus sp*
Papilio demoleus (Linnaeus,1758)	Lime butterfly	VC	*Citrus sp, Murraya paniculata*
Papilio crino (Fabricius, 1793)	Common Banded Peacock	VR	-
Pachliopta aristolochiae (Fabricius,1775)	Common Rose	VC	*Aristolochia bracteolata*
Pachliopta hector (Linnaeus,1758)	Crimson Rose	VC	*Aristolochia bracteolata*
Troides minos (Cramer, 1779)	Southern Bird wing	VR	*Aristolochia griffithi*
Family: Hesperiidae, Subfamily: Coeliadinae, Pyrginae, Hesperiinae			
Badamia exclamationis (Fabricius,1775)	Brown awl	R	*Terminalia catappa*
Spialia galba (Fabricius,1793)	Indian skipper	C	*Hibiscus, Sida rhombifolia*
Gomalia elma (Trimen,1862)	African marbled skipper	UC	*Abutilon indicum*
Taractrocera maevius (Fabricius,1793)	Common grass dart	UC	*Poaceae*
Telicota sp	Dart	C	*Poaceae*
Borbo cinnara (Wallace,1866)	Rice swift	C	*Poaceae, Brachiaria mutica*
Suastus gremius (Fabricius,1798)	Indian palm bob	UC	*Arecaceae, Caryota urens*
Gangara thyrsis (Fabricius,1775)	Giant red eye	R	*Arecaceae, Caryota urens*
Iambrix salsala (Moore,1866)	Chestnut bob	C	*Poaceae, Brachiaria mutica*
Udaspes folus (Cramer,1775)	Grass demon	R	*Curcuma aromatica*
Family: Pieridae, Subfamily: Coliadinae, Pierinae			
Eurema hecabe (Linnaeus,1758)	Common grass yellow	VC	*Albizia saman, Cassia fistula, Peltophorum pterocarpum, Pithecellobium dulce*
Catopsilia pomona (Fabricius,1775)	Common emigrant	VC	*Cassia fistula*
Catopsilia pyranthe (Linnaeus,1758)	Mottled emigrant	VC	*Cassia fistula, Senna occidentalis*
Colotis amata (Fabricius,1775)	Small salmon arab	C	-
Colotis danae (Fabricius,1775)	Crimson tip	UC	*Capparis divaricate*
Colotis etrida (Boisduval,1836)	Small orange tip	UC	-
Ixias marianne (Cramer,1779)	White orange tip	C	*Capparis divaricata*
Ixias pyrene (Linnaeus,1764)	Yellow orange tip	C	*Capparis divaricata*
Hebomia glaucippe (Butler,1898)	Great orange tip	C	*Capparis sp*
Pareronia hippia (Fabricius,1787)	Common wanderer	C	*Capparis sp*
Appias libythea (Fabricius,1775)	Striped albatross	UC	*Capparis sp*
Cepora nerissa (Fabricius,1795)	Common gull	VC	*Capparis sp*
Delias eucharis (Drury,1773)	Common Jezebel	UC	*Loranthus longiflorus*

Table continue

Table 1: List of butterflies observed in the Botanical Garden, TNAU

Scientific Name	Common Name	Status	Host plants in Garden
Leptosia nina (Fabricius,1793)	Psyche	VC	*Capparis sp*
Belenois aurota (Fabricius,1793)	Pioneer	VC	*Capparis sp*
Family: Lycaenidae, Subfamily: Miletinae, Curetinae, Theclinae, Polyommatinae			
Spalgis epeus (Westwood,1851)	Apefly	R	*Coccidae (Insecta)*
Curetis thetis (Drury,1773)	Indian Sunbeam	R	*Abrus precatorius, Pongamia pinnata*
Arhopala amantes (Hewitson,1862)	Large oak blue	UC	*Terminalia catappa, Syzygium cumini, Lagerstroemia speciosa*
Zesius chrysomallus (Huebner,1819)	Redspot	R	*Terminalia catappa, Cassia fistula,*
Rathinda amor (Fabricius,1775)	Monkey puzzle	C	*Mangifera indica, Ixora sp*
Deudorix isocrates (Fabricius,1793)	Guava blue	C	*Tamarindusindica, Citrus sp*
Deudorix epijarbas (Moore,1858)	Cornelian	C	-
Rapala iarbus (Fabricius,1787)	Indian red flash	R	*Terminalia catappa, Acacia leucophloea*
Spindasis vulcanus (Fabricius,1775)	Common Silverline	C	*Cadaba fruticosa, Cassia fistula, Clerodendrum indicum*
Spindasis schistacea (Moore,1881)	Plumbeous silver line	C	*Quisqualis indica*
Spindasis ictis (Hewitson,1865)	Common shot silverline	UC	*Senna sp*
Castalius rosimon (Fabricius,1775)	Common pierrot	C	*Ziziphus sp*
Leptotes plinius (Fabricius, 1793)	Zebra blue	C	*Abrus precatorius, Plumbago zeylanica*
Jamides celeno (Cramer,1775)	Common Cerulean	C	*Abrus precatorius, Pongamia pinnata*
VC-Very Common, C-Common, UC-Uncommon, R-Rare, VR-Very Rare			
Catochrysops strabo (Fabricius, 1793)	Forget-me-not	C	*Acacia sp, Pongamia pinnata*
Lampides boeticus (Linnaeus,1767)	Pea blue	VC	*Fabaceae, Abrus precatorius, Pongamia pinnata*
Tarucus nara (Kollar,1844)	Stripped pierrot	VR	*Ziziphus jujube*
Zizeeria karsandra (Moore,1865)	Dark grass blue	VC	*Amaranthus viridis, Oxalis corniculata*
Pseudozizeeria maha (Kollar,1844)	Pale grass blue	UC	*Tephrosia purpurea, Oxalis corniculata*
Freyeria trochylus (Freyer,1845)	Grass jewel	C	*Oxalis corniculata, Indigofera sp*
Zizina otis (Fabricius,1787)	Lesser grass blue	C	*Amaranthus viridis, Tribulus terrestris*
Zizula hylax (Fabricius, 1775)	Tiny grass blue	VC	*Ruellia tuberosa, Lantana, Tribulus terrestris*
Azanus jesous (Guerin Meneville,1847)	African Babul blue	C	-
Talicada nyseus (Guerin Meneville,1843)	Red pierrot	R	*Bryophyllum pinnatum*
Euchrysops cnejus (Fabricius, 1798)	Gram blue	VC	*Fabaceae*
Acytolepis puspa (Horsfield,1828)	Common hedge blue	VC	*Peltophorum pterocarpum*
Chilades pandava (Horsfield,1829)	Plains cupid	VC	*Cycas revoluta*
Chilades lajus (Cramer,1782)	Lime blue	VC	*Citrus, Mangifera indica*
Azanus jesous (Guerin Meneville,1847)	African Babul blue	C	-
Family: Nymphalidae, Subfamilies: Danainae, Charaxinae, Satyrinae, Heliconinae, Limenitinae, Biblidinae, Nymphalinae			
Tirumala limniace (Cramer,1775)	Blue tiger	VC	*Vallaris solanacea, Calotrophis gigantea, C. procera*
Tirumala septentrionis (Butler,1874)	Dark blue tiger	VC	*Vallaris solanacea*
Danaus genutia (Cramer,1779)	Striped tiger	VC	*Ceropegia sp*
Danaus chrysippus (Linnaeus,1758)	Plain tiger	VC	*Calotrophis gigantea, C. procera*
Euploea sylvester (Fabricius,1793)	Double branded crow	C	*Ficus sp*
Euploea klugii (C.&R. Felder,1865)	Brown King Crow	C	*Ficus sp*
Euploea core (Stoll,1780)	Common crow	C	*Adenium obesum, Nerium oleander, Ficus sp*
Charaxes solon (Fabricius,1773)	Black Rajah	R	*Pithecellobium dulce, Tamarindus indica*
Melanitis leda (Linnaeus,1758)	Common evening brown	VC	*Poaceae, Bambusa sp, Pennisetum sp*
Lethe europa (Fabricius,1775)	Bamboo Tree Brown	VR	*Poaceae, Bambusa sp*
Mycalesis perseus (Fabricius,1775)	Common Bush brown	VC	*Poaceae*
Ypthima ceylonica (Hewitson,1865)	White four ring	VC	*Poaceae, Setaria barbata*
Ypthima asterope (Klug,1832)	Common three ring	C	*Poaceae*
Elymnias caudata (Butler,1871)	Tailed palmfly	C	*Areca catechu, Caryota urens*
Acraea violae (Fabricius,1793)	Tawny coster	VC	*Turnera subulate*

Table continue

Table 1: List of butterflies observed in the Botanical Garden, TNAU

Scientific Name	Common Name	Status	Host plants in Garden
Phalanta phalantha (Drury,1773)	Common leopard	C	-
Neptis hylas (Linnaeus,1758)	Common Sailer	C	*Fabaceae, Malvaceae*
Euthalia aconthea (Cramer,1777)	Common baron	C	*Mangifera indica*
Ariadne ariadne (Linnaeus,1763)	Angled castor	UC	*Ricinus communis*
Ariadne merione (Cramer,1777)	Common castor	C	*Ricinus communis*
Byblia illthiya (Drury,1773)	Joker	R	-
Vanessa cardui (Linnaeus,1758)	Painted lady	R	-
Junonia orithiya (Linnaeus,1758)	Blue pansy	UC	*Justicia sp, Ruellia tuberosa*
Junonia hierta (Fabricius,1798)	Yellow pansy	VC	*Justicia sp, Barleria sp, Ruellia tuberose, Asystasia sp*
Junonia iphita (Cramer,1779)	Chocolate pansy	VC	*Barleria sp, Justicia sp, Ruellia tuberosa,*
Junonia atlites (Linnaeus,1763)	Grey pansy	VR	*Barleria sp*
Junonia almanac (Linnaeus,1758)	Peacock pansy	C	*Barleria sp, Poaceae*
Junonia lemonias (Linnaeus,1758)	Lemon pansy	VC	*Barleria sp, Justicia sp*
Hypolimnas bolina (Linnaeus,1758)	Great eggfly	UC	*Alternanthera sessilis, Portulaca oleracea,*
Hypolimnas misippus (Linnaeus,1764)	Danaid eggfly	C	*Asystasia gangetica, Portulaca oleracea*

VC-Very Common, C-Common, UC-Uncommon, R-Rare, VR-Very Rare

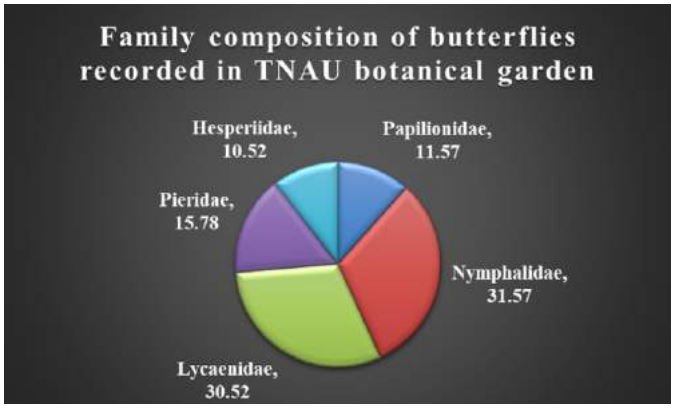

Fig. 1: Family-wise distribution of butterflies in TNAU Botanical Garden, Coimbatore.

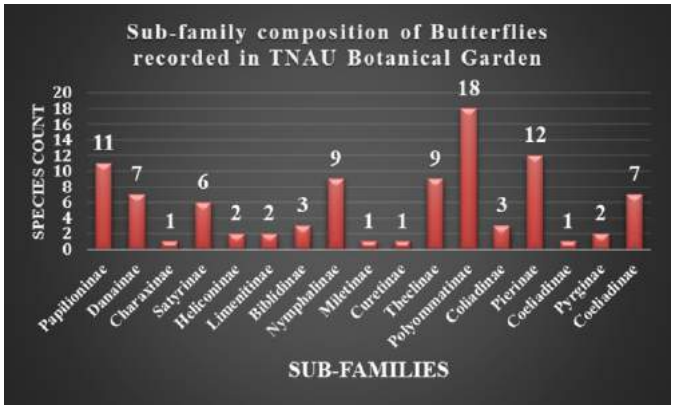

Fig. 2: Subfamily-wise distribution of butterflies in TNAU Botanical Garden, Coimbatore.

Fig. 3: Family Papilionidae: *Papilio polymnestor; Graphium nomius; Papilio demoleus; Papilio polytes; Pachliopta hector; Papilio helenus; Papilio crino; Graphium doson; Troides minos; Graphium agamemnon; Pachliopta aristolochiae.*

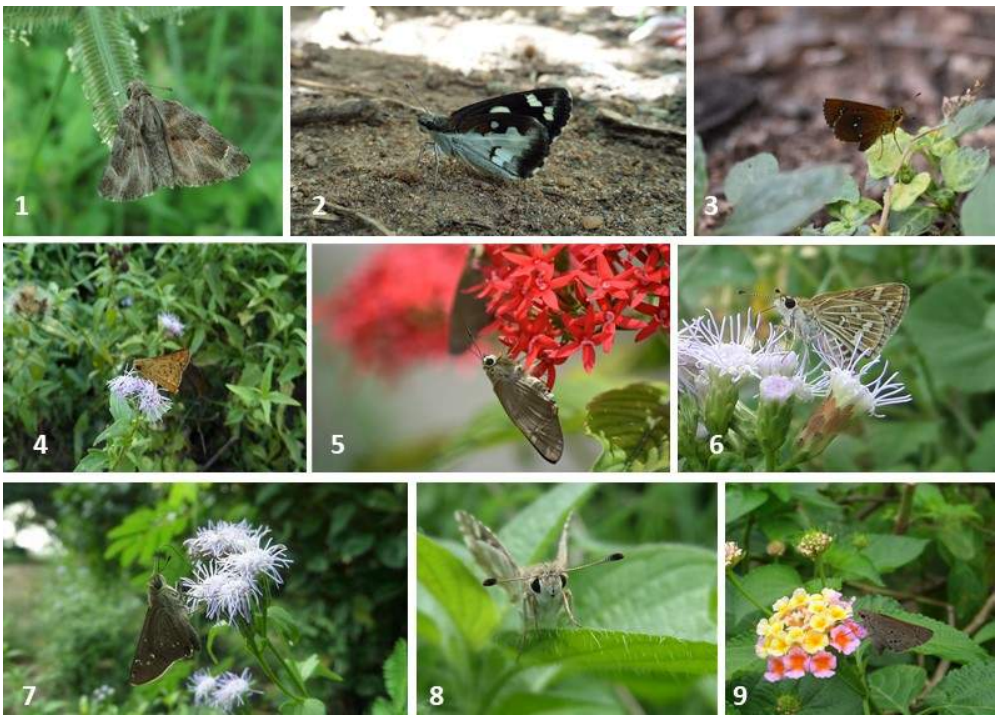

Fig. 4: Family Hesperiidae: *Gomalia elma*; *Udaspes folus; Iambrix salsala; Telicota sp; Badamia exclamationis; Taractrocera maevius; Borbo cinnara; Spialia galba; Suastus gremius.*

Fig. 5: Family Pieridae: *Delias eucharis; Catopsilia pyranthe; Hebomoia glaucippe; Colotis danae; Cepora nerissa; Pareronia hippia; Catopsilia pomona; Colotis amata; Eurema hecabe; Ixias marianne; Leptosia nina; Belenois aurota.*

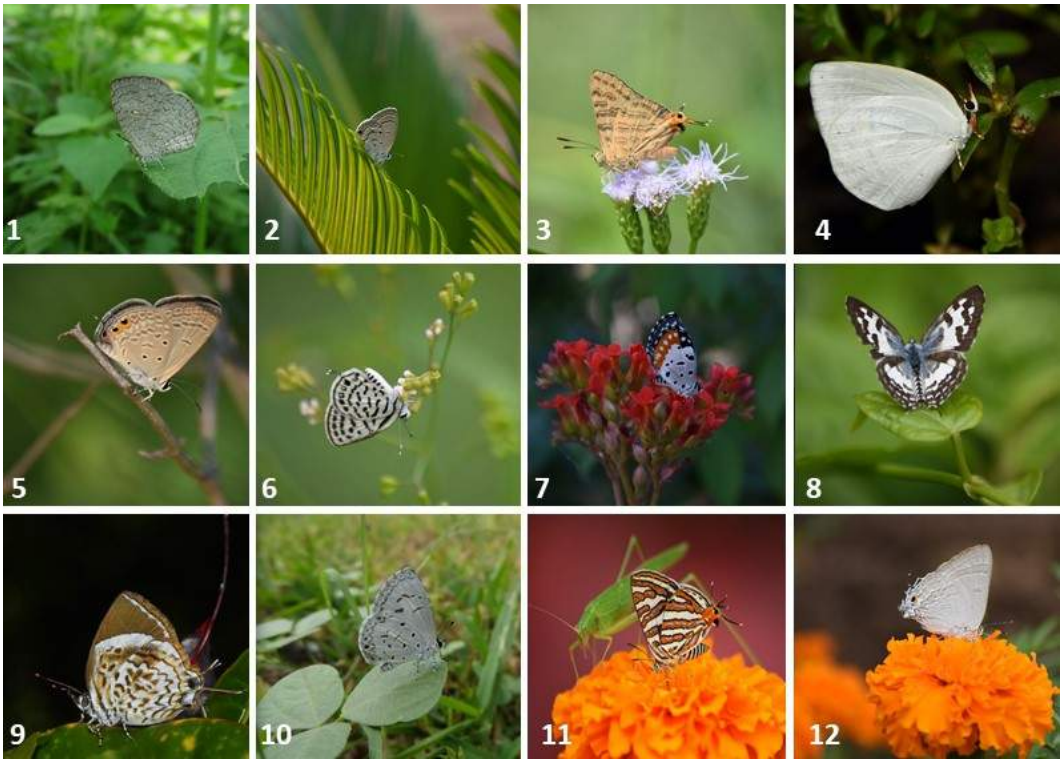

Fig. 6: Family Lycaenidae: *Spalgis epeus; Chilades pandava; Spindasis ictis; Curetis thetis; Euchrysops cnejus; Tarucus nara; Talicada nyseus; Castalius rosimon; Rathinda amor; Acytolepis puspa; Spindasis schistacea; Deudorix isocrates; Lampides boeticus; Freyeria putli; Azanus jesous; Chilades lajus; Jamides celeno; Zizula hylax; Zizeeria karsandra; Leptotes plinius; Chilades trochylus; Catochrysops strabo; Deudorix epijarbas; Rapala iarbus.*

Fig. 7: Family Nymphalidae: *Byblia illthiya; Junonia almana; Ypthima ceylonica; Danaus chrysippus; Tirumala limniace; Tirumala septentrionis; Hypolimnas missipus; Danaus genutia; Junonia hierta; Elymnias caudata; Euploea sylvester; Junonia atlites; Lethe europa; Ariadne merione; Vanessa cardui; Melanitis leda*; *Hypolimnas bolina; Junonia orithiya; Neptis hylas; Phalanta phalantha; Junonia iphita.*

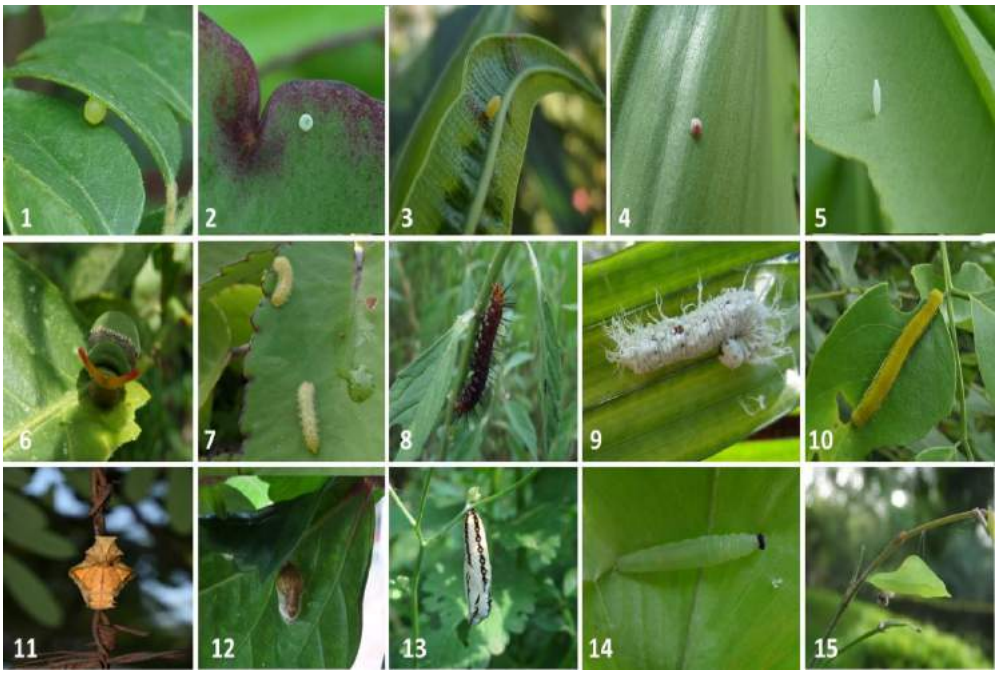

Fig. 8: Early stages of some butterfy eggs: Papilionidae -*Papilio polytes;* Lycaenidae-*Talicada nyseus;* Nymphalidae-*Euploea core;* Hesperiidae-*Udaspes folus*; Pieridae-*Catopsilia pomona*. **Caterpillars:** Papilionidae -*Papilio demoleus;* Lycaenidae-*Talicada nyseus;* Nymphalidae-*Acraea terpiscore;* Hesperiidae-*Gangara thyrsis;* Pieridae-*Catopsilia pomona.* **Chrysalis:** Papilionidae -*Troides minos;* Lycaenidae-*Spalgis epius;* Nymphalidae- *Acraea terpiscore;* Hesperiidae-*Udaspes folus;* Pieridae-*Eurema hecabe.*

reported by Nimbalkar *et al.* (2011) in a study conducted in Pune district regarding butterfly diversity in relation to nectar food plants. In *Tridax procumbens*, the capitulum with a long stalk attracts foragers. The capitulum consists of numerous florets arranged in an aggregate manner with vertical orientation. The ray and disc florets both attract butterflies as capitulum offers good platform for exploring florets (Aluri *et al.,* 2013). Diversity and abundance of butterflies were strongly influenced by nectar sources. Gutierezz and Mendez (1995) elucidated that, the abundance of butterflies were indeed related to the availability of food plants. During the presence of nectar sources (*Zinnia elegans, Cosmos sp., Eupatorium sp.*) the butterfly activity was considerable and there was a decline in butterfly sightings during non-availability of nectar plants. Preferences of nectar sources were also studied. *Eupatorium odoratum* in bloom attracted a lot of butterflies and in particular, Silver lines of Lycaenidae showed more preference to this plant. Occurrence of Silver line butterflies decreased when the plants were pruned for regrowth.

Species with shorter tongues do not visit flowers with deeper corolla tubes. Hence, short tongued Lycaenids like Silver lines are inclined towards Asteraceae members with short corolla tubes like *Eupatorium odoratum* and *Solidago canadensis* (Corbet, 2000). Monkey puzzle butterfly (*Rathinda amor*) and Ape fly (Spalgis epeus) were strongly attracted to Noni flower nectar. Sightings of butterflies became rare when annual nectar plants weren't raised in the garden. Annuals like *Cosmos sulphureus* and *Zinnia elegans* attracted many members of Swallowtails (Papilionidae) and Brush-footed butterflies (Nymphalidae). Large butterflies preferred flowers like Zinnia and Cosmos as their petals aid in landing and support. It is very rare to see them around small flowers like *Tridax procumbens.* Lycaenidae, Pieridae and Hesperiidae family members preferred both *Zinnia elegans* and *Tridax procumbens*. In comparison with herbs and shrubs, tree flowers are rarely preferred as a nectar source. Considering host plants, polyphagous caterpillars survived in several hosts and their population was stable in spite of removal of certain hosts. Monophagous caterpillars failed to survive and were not observed till the restoration of host plants. Also, the butterfly activity was considerable after the monsoon period and tended to decline during the dry season.

Post monsoon triggered the growth of young shoots which positively promoted the growth of butterfly caterpillars. Host plants for 88 butterflies have already been maintained in the garden. Planting host plants of *Papilio crino (Chloroxylon swietenia), Colotis amata (Toddalia asiatica, Salvadora sp.), Colotis etrida (Cadaba fruticosa), Deudorix epijarbas (Sapindus sp), Azanus jesous (Acacia leucophloea), Phalanta phalantha (Flacourtia indica), Byblia ilithiya (Tragia involucrata), Vanessa cardui (Malva sp* and *Lablab purpureus)* will increase the abundance of rare and uncommon visitors. In urban area, the presence of 95 species in a single place is highly considerable. This study provides the required information for raising suitable host and nectar plants. Planting and maintenance of appropriate host plants and ensuring the availability of prominent nectar sources throughout the year will aid in better conservation of these colourful creatures.

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Add-on Information

Authors' contribution: G. Vinithashri: Field studies, data collection and manuscript preparation; **J.S. Kennedy:** Guidance for conducting the work.

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Probing cellulolytic yeast from forest ecosystem for the saccharification of Napier fodder biomass

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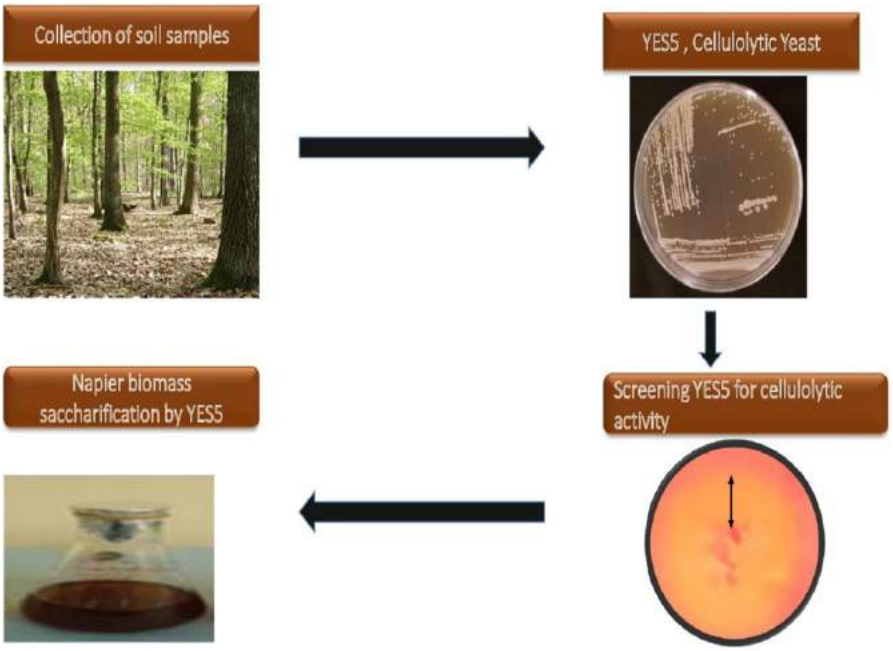
Abstract

Aim: This study aimed to search for novel cellulolytic isolates with high cellulase titre for the production of fuels and chemicals.

Methodology: The yeast isolate YES5 isolated from the forest soil was screened for cellulase production. The cellulase activity of YES5 was optimized via RSM. The saccharification potential of YES5 using Napier biomass as substrate was evalauted.

Results: The maximum cellulase activity obtained after optimizing pH, temperature, and incubation period was 35.70 U. A reliable statistical model was developed for maximizing the cellulase activity in YES5 *Trichosporon asahii*. The cellulase activity was 23.87U, when carbon source in CMC medium was replaced by Napier biomass. The maximum saccharification potential of 33.15% was observed on 3rd day.

Interpretation: The study of optimizing the media composition of *Trichosporon asahii* cellulase using Napier biomass, a natural source of carbon for maximizing the cellulase production via RSM, is first of its kind.



Key words: Cellulase, Napier biomass, Saccharification, *Trichosporon asahii*

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Introduction

As the world population is increasing at an alarming rate, the energy consumption ratio is also rising. Forests and people are tightly coupled in this country (Baboo *et al.,* 2017). Studies have reported that millions of people reside within or close to forests and harvest forest products (Davidar *et al.,* 2010; Gosain *et al.,* 2015; Bargali and Bargali 2016). The activities occurring in the forests include exploitation through commercial logging, seasonally set forest fires, fuel wood removal, charcoal production, cattle grazing, pruning and land clearing for agricultural activities (Bhuyan *et al.,* 2003; Bargali *et al.,* 2019). These disturbances have changed the forest composition, structure and reduced species diversity as well as resources by restricting size of forest patches (Sagar and Singh 2005; Karki *et al.,* 2017). Continued increase in human population together with livestock populations, the pressure on these forests has increased tremendously and consequently resulting in reduced carrying capacity of these forests (Bargali *et al.,* 2018; Manral *et al.,* 2020). The increasing population and reduced resources have increased the gap between demand and supply of ecosysytem services (Bargali *et al.,* 1992a, b; 1993; Gosain *et al.,* 2015).

Nonrenewable resources may not be sufficient to meet out the energy requirement of the society so there is a swift in the utilization of resources from nonrenewable to renewable for the production of value added compounds (Gaurav *et al.,* 2017; Valliammai *et al.,* 2021). The environment concerning factors which have paved the path for the production of microbial cellulases are global warming and greenhouse effects (Vanhala *et al.,* 2016). In the current era, microbes play a key role in biomass conversion (Shariq and Sohail, 2018). The effective roles contributed by microbes in bio refinery concept are pretreatment, hydrolysis, detoxification and fermentation. The performance of microbes varies according to the desired yield (Dien *et al.,* 2003). Application of microbes or microbial enzymes for the pretreatment of lignocellulosic biomass is gaining momentum in the industry. Cellulases are inducible enzymes which can be produced by microbes during their growth on cellulosic material (Lee and Koom, 2001). Structurally fungal cellulases are simpler than bacterial cellulase systems (Artzi *et al.,* 2015).

Different kinds of microbes involved in the production of cellulase are aerobic, anaerobic, mesophilic or thermophilic. Among them, *Clostridium, Cellulomonas*, *Thermomonospora, Trichoderma* and *Aspergillus*. They are the most extensively studied cellulase producers (Sukumaran *et al.,* 2005). *Trichosporon asahii* belongs to genus *Trichosporon*, a genus of anamorphic fungi of family *Trichosporonaceae*. Limited studies are available with regard to cellulolytic potential of *Trichosporon asahii*. The predominant yeast species found in waste biomass conversion to bioproducts reported so far are *Candida tropicalis, Candida intermedia, Candida parapsilosis, Pichia guilliermondii, Saccharomyces cerevisiae* and *Trichosporon asahii* (Schwan *et al.,* 2007). Yeasts like *Candida tropicalis* are involved saccharification of rice straw, corn-cob (Guo *et al.,* 2013). Exploring the nature of cellulase activity of *Trichosporon asahii* in Napier (*Pennisetum purpureum*) fodder biomass conversion into ethanol is first of its kind. Cellulase activity can be observed in diverse group of microbes while utilizing cellulosic materials (Lu *et al.,* 2010). Cellulase activity in microbes can be improved by nutritional and process parameters during fermentation (Rasul *et al.,* 2015). Response surface methodology is an efficient technique utilized for optimization of fermentation processes (Valliammai *et al.,* 2020). Mathematical models are proven tools in the optimisation of process parameters during fermentation (Imamoglu and Sukan, 2013). Napier fodder (*Pennisetum purpureum*) is a fast growing perennial crop widely observed in tropical and subtropical regions of the world. It has a higher yield per unit area and the biomass productivity is also commendable (Woodard and Prine, 1993) High cellulose content in Napier fodder makes it a promising substrate for the biobased economy. In this study, yeast YES5 Trichosporon asahii was isolated from the forest soil sample. The cellulase activity of YES5 using Napier biomass as carbon source was optimized via RSM. The study of standardising the cellulase activity of YES5 for the Napier biomass saccharification is a novel approach.

Materials and Methods

Isolation and screening of cellulolytic yeasts from forest ecosystem: Forest soil samples were collected from the Eastern Ghats region (N 10. 29 363◦) (E 77. 70 674◦) of Thandikudi, Tamil Nadu, India. Isolation was initiated via serial dilution technique followed by pour plating. Ten gram of soil sample was suspended in 99 ml of water and used for isolation of cellulolytic yeast using carboxymethyl cellulose agar medium and incubated at 28°C for 2-3 days (Hendricks *et al.,* 1995). The appeared colonies were purified and then screened for cellulase activity via congo-red agar medium (Hankin and Anagnostakis, 1977). Among different yeast isolates, YES5 showed maximum cellulase activity and, hence, was selected for further study.

Identification of cellulolytic yeast isolate YES5: Cellulolytic yeast isolate was cultivated in CMC agar medium, and DNA was extracted (Harju *et al.,* 2004). Nearly-full length 18S rRNA gene was amplified from the genomic DNA of YES5 isolate using a forward primer as NL-1 (5' GCA TAT CAA TAA GCG GAG GAA AAG 3') and reverse primer as NL-4 (5' GGT CCG TGT TTC AAA GAC GG 3') primers. The PCR product was sequenced through ABI prism terminator cycle sequencing ready reaction kit, and electrophoresis of the products were carried out on an Applied Biosystems (Model 3100) automated sequencer (M/s. Yaazh Genomics, Coimbatore, India). Isolate YES5 exhibited 96.96 % similarity to *Trichosporon asahii* MN368077. The 18S rRNA gene sequence was submitted to NCBI with accession number MK640632.

Cellulase assay: *Trichosporon asahii* YES5 was cultivated in CMC broth at 28◦C for 2 days, and the culture supernatant was collected by centrifugating at 13000 rpm for 8 min. A 200 µl of culture supernatant was mixed with 200 µl of 1% CMC solution and incubated at 50°C for 30 min. DNS reagent was added and incubated at 100°C for 10 min. The absorbance was read at 575 nm against reagent blank. In this study, one unit of enzyme activity refers to the release of 1 µmol of reducing sugar per min per ml (Wood and Bhat, 1988).

Effect of carbon source in cellulase activity: The influence of different carbon sources was determined by substituting the carbon sources *viz.,* fructose, galactose, sucrose, lactose, starch, and CMC individually in place of carbon source in the medium. About 5µl of 24-hr-old culture (7 x 10^8 CFU ml^{-1}) was inoculated in 100 ml medium supplemented with different carbon sources and incubated at 28°C for 24 hr. The culture supernatant from each source was collected and assayed for cellulase activity (Wood and Bhat, 1988).

Effect of pH and temperature on cellulase activity: Cellulase activity was determined at different incubation period, temperature and initial pH. Five microliter of 24-hr-old culture (7 x 10^8 CFU ml^{-1}) of YES5 *Trichosporon asahii* was inoculated into CMC broth and incubated at 28°C for different time period (5 days). The day at which maximum cellulase activity observed was considered for further optimizing the pH range (4.5 to 8.5 with an increment of 1.0) and temperature range (25°C to 40°C with an increment of 5°C).

Statistical optimization of cellulase activity using Napier biomass as a carbon source: For maximizing cellulase production, RSM was employed with CCD. The selected parameters and their range are provided in Table1. A total of 78 experiments were performed with different combinations (Table 2). Cellulase assay was performed as described earlier. The response value (Y) in each trial is the average of duplicates. The design expert trial version 12 was used for the development of design matrix and statistical analysis of the data. Regression analysis was performed to estimate the response function as a second-order polynomial (Wang *et al.,* 2011):

$$Y = \beta 0 + \_\beta iXi + \_\beta ijXiXj + \_\beta iiXi2$$

Where, *Y* is the predicted response, β0 is the intercept term, βi is the linear coefficient, βij is the quadratic coefficient and βii is the interaction coefficient. Analysis of variance (ANOVA) was adopted to know the significance of the model. The quality of polynomial model equation was judged statistically through coefficient of determination (R^2) and adjusted R^2. Overall significance was evaluated through Fishers test. Three-dimensional plots illustrated the response for each interaction. In order to validate the model predicted by the software, experiments were performed in shake-flasks. Physical parameters were kept at optimum levels. Cellulase assay was performed as described earlier.

Compositional analysis of Napier biomass: The compositional analysis of Napier biomass was determined as per the standard protocols of NREL, 2004 (Sluiter *et al.,* 2012).

Saccharification of Napier biomass: The leftover Napier fodder in the field was collected and dried in a tunnel drier to get a brittle texture. The dried powder was sieved through a 50µ sieve. One gram of powder was added to 100ml of medium containing optimized levels of nitrogen source and mineral salts (1g K_2HPO_4, 0.5g $MgSO_4$, 0.5gNaCl,1.0g peptone, 0.25g $(NH_4)2SO_4$) and autoclaved at 110°C, 15lbs pressure for 20 min. 5 µl of 24 h old culture (7 x 10^8 CFU ml^{-1}) *Trichosporon asahii* was inoculated and allowed for saccharification at 30°C and 120 rpm for 5 days. Samples were taken at an interval of 24 hr to monitor sugar release. The experiments were performed in triplicates. The percentage of saccharification was noted as per the standard protocols of NREL, 2004 (Uma *et al.*, 2010).

Results and Discussion

The yeast isolate YES5 was creamy, glossy colonies with a cell morphology of globose shape present singly, multilateral budding with ascospores. The isolate YES5 was identified based on 18S rRNA sequencing as *Trichosporon asahii*. Certain yeast strains like *Pichia stipitis, Saccharomyces cerevisiae*, and *Kluyveromyces fagilis* were reported as good ethanol producers from different types of sugars (Mussatto *et al.,* 2012). Though there are many cellulolytic organisms, researchers are still exploring novel microbes for biomass conversion into value-added products. A clear zone of hydrolysis was observed around the colony of isolate YES5 suggesting cellulose hydrolyzing nature of YES5. (Fig. 1).

The carbon sources used in the assay were fructose, galactose, sucrose, lactose, starch, and CMC. The YES5 yeast

Table 1: Factors employed in the RSM experiment for optimum cellulase activity

Factors	+ alpha	- alpha	Low value	High value
Napier biomass	2.174	17.825	5	15
Peptone	2.174	17.825	5	15
NaCl	0.043	0.356	0.1	0.3
$MgSO_4$	0.065	0.534	0.15	0.45
$(NH_4)_2SO_4$	0.043	0.356	0.1	0.3

Table 2: Optimization of nutrient components for maximum cellulase activity

Factor 1 A:Napier biomass	Factor 2 B:Peptone	Factor 3 C:Dipotassium hydrogen phosphate	Factor 4 D:Magnesium sulphate	Factor 5 E:Ammonium sulphate	Factor 6 F:Sodium chloride	Observed Cellulase activity (U ml^{-1})	Predicted Cellulase activity (U ml^{-1})
15	15	0.1	0.15	0.3	0.5	19.03	19.66
5	15	0.1	0.15	0.3	1.5	17.79	16.43
10	10	0.2	0.3	0.2	1	23.36	23.87
5	15	0.1	0.15	0.1	0.5	17.69	16.16
15	15	0.3	0.45	0.1	1.5	19.25	19.04
5	15	0.3	0.15	0.3	0.5	17.38	16.67
5	5	0.3	0.45	0.1	0.5	15.82	15.53
15	5	0.3	0.15	0.3	0.5	16.19	16.38
5	15	0.3	0.45	0.3	1.5	15.92	16.02
10	10	0.2	0.3	0.2	0.217458	23.87	23.55
10	10	0.2	0.3	0.2	1.78254	23.45	23.50
15	5	0.3	0.45	0.1	0.5	17.02	17.19
5	15	0.3	0.15	0.1	1.5	15.88	16.09
5	5	0.3	0.15	0.1	0.5	15.04	15.37
5	15	0.1	0.45	0.3	0.5	15.77	16.05
5	5	0.3	0.15	0.3	0.5	15.00	14.91
15	5	0.1	0.15	0.3	1.5	17.82	17.59
15	15	0.3	0.45	0.3	0.5	19.89	19.55
5	15	0.3	0.45	0.3	0.5	15.89	16.65
5	5	0.3	0.45	0.1	1.5	15.02	15.31
15	5	0.3	0.15	0.1	0.5	17.26	16.87
17.8254	10	0.2	0.3	0.2	1	18.27	18.27
5	5	0.1	0.45	0.3	0.5	15.05	14.67
5	15	0.3	0.45	0.1	0.5	17.21	16.56
10	10	0.2	0.0652373	0.2	1	22.18	22.22
5	5	0.1	0.15	0.3	0.5	15.05	15.07
15	5	0.3	0.15	0.3	1.5	16.87	16.88
10	10	0.2	0.3	0.0434915	1	23.45	22.84
15	5	0.1	0.15	0.1	0.5	17.01	17.18
15	15	0.1	0.15	0.1	1.5	18.94	19.35
10	2.17458	0.2	0.3	0.2	1	16.93	17.73
5	5	0.1	0.45	0.1	1.5	15.00	14.97
15	5	0.1	0.45	0.1	1.5	17.01	17.27
15	15	0.1	0.15	0.1	0.5	19.32	19.30
15	15	0.3	0.15	0.3	1.5	19.44	19.39
5	5	0.3	0.15	0.1	1.5	16.59	15.48
15	5	0.3	0.45	0.1	1.5	17.01	17.26
15	5	0.1	0.45	0.3	0.5	16.98	16.66
15	15	0.1	0.15	0.3	1.5	19.24	19.82
15	15	0.3	0.15	0.3	0.5	19.99	19.42
15	5	0.3	0.45	0.3	0.5	16.83	16.62
10	10	0.0434915	0.3	0.2	1	23.22	22.51
15	5	0.1	0.45	0.1	0.5	16.78	17.02
5	5	0.3	0.45	0.3	1.5	15.02	14.88
15	15	0.3	0.45	0.1	0.5	19.06	19.49
15	5	0.3	0.15	0.1	1.5	17.27	17.27
2.17458	10	0.2	0.3	0.2	1	14.58	14.31
5	5	0.1	0.45	0.1	0.5	15.02	14.99
10	10	0.2	0.3	0.2	1	23.43	23.87
5	15	0.3	0.45	0.1	1.5	16.22	15.82
5	15	0.3	0.15	0.3	1.5	16.11	16.36
15	15	0.1	0.45	0.3	0.5	19.05	19.31

Table continiue

Table 2: Optimization of nutrient components for maximum cellulase activity

Factor 1 A:Napier biomass	Factor 2 B:Peptone	Factor 3 C:Dipotassium hydrogen	Factor 4 D:Magnesium sulphate	Factor 5 E:Ammonium sulphate	Factor 6 F:Sodium chloride	Observed Cellulase activity (U ml^{-1})	Predicted Cellulase activity (U ml^{-1})
15	5	0.1	0.45	0.3	1.5	16.92	17.02
15	15	0.3	0.45	0.3	1.5	19.04	19.20
10	17.8254	0.2	0.3	0.2	1	21.34	20.27
10	10	0.2	0.534763	0.2	1	22.12	21.81
5	15	0.3	0.15	0.1	0.5	15.17	16.50
5	5	0.1	0.45	0.3	1.5	15.02	14.75
5	15	0.1	0.45	0.3	1.5	15.08	15.60
15	15	0.1	0.45	0.3	1.5	19.67	19.14
5	5	0.1	0.15	0.1	1.5	15.06	15.62
15	15	0.3	0.15	0.1	1.5	19.02	19.15
15	5	0.1	0.15	0.3	0.5	16.94	16.91
5	5	0.1	0.15	0.3	1.5	15.04	15.48
5	5	0.3	0.45	0.3	0.5	15.08	14.99
15	15	0.1	0.45	0.1	0.5	19.03	19.03
15	5	0.1	0.15	0.1	1.5	18.68	17.77
5	5	0.3	0.15	0.3	1.5	15.09	15.12
5	5	0.1	0.15	0.1	0.5	15.02	15.32
10	10	0.2	0.3	0.356508	1	22.43	22.77
5	15	0.1	0.45	0.1	1.5	15.05	15.18
15	15	0.1	0.45	0.1	1.5	18.85	18.76
15	5	0.3	0.45	0.3	1.5	16.75	16.79
5	15	0.1	0.45	0.1	0.5	15.10	15.73
10	10	0.356508	0.3	0.2	1	22.16	22.60
5	15	0.1	0.15	0.3	0.5	16.01	16.55
15	15	0.3	0.15	0.1	0.5	19.28	19.28
5	15	0.1	0.15	0.1	1.5	15.02	15.93

Table 3: Cellulolytic potential of *Trichosporon asahii* YES5 in synthetic and natural source of carbon

Constant parameters	Variable parameters	Range	Optimum	Maximum activity
Basal medium+ Incubation time+ Temperature+ pH	Synthetic carbon sources		CMC	21.23 U ml^{-1}
Basal medium+ CMC+ Temperature+ pH	Incubation time	1-5 days	3rd day	29.90 U ml^{-1}
Basal medium+ CMC+ Optimum Incubation Time+ pH	Temperature	25-40◦C	30◦C	35.70U ml^{-1}
Basal medium+ CMC+ Optimum Incubation Time+ Optimum temperature	pH	4.5-8.5	6.5	34.49 U ml^{-1}
Optimum Incubation Time+ Optimum	Napier biomass	2.174-17.825 g l^{-1}	10 g l^{-1}	
Temperature+	Peptone	2.174-17.825 g l^{-1}	10 g l^{-1}	23.87
Optimum pH	NaCl	0.043-0.356 g l^{-1}	0.2 g l^{-1}	U ml^{-1}
	$MgSO_4$	0.065-0.534 g l^{-1}	0.3 g l^{-1}	
	$(NH_4)2SO_4$	0.043-0.356 g l^{-1}	0.2 g l^{-1}	
	K_2HPO_4	0.2174- 1.7825 g l^{-1}	0.217 g l^{-1}	

isolate showed a positive response towards all the tested carbon sources, however, the impact of CMC as a source of carbon showed much higher cellulase activity compared to other carbon sources. The cellulase activity of YES5 isolate using CMC as a substrate was 21.22 U. (Fig. 2). Lignocellulosic biomass acts as a source of carbon for the microorganisms during the fermentation process (Battaglia *et al.,* 2011). The most important carbon source in biomass is cellulose and is converted into value-added

products by the enzyme cellulase (Lu *et al*., 2013; Valliammai *et al.,* 2021). Maximum cellulase activity was observed by optimizing the factors like incubation period, temperature, and pH. (Fig. 3A-C). The cellulase activity was observed throughout the incubation period; however, it was maximum on day 3 (29.90 U). Though cellulase activity was observed 25-45°C, the optimum temperature for obtaining maximum cellulase activity was 30°C (35.70 U). While increasing the temperature, cellulase activity decreased. Among the various pH tested, the optimum pH for cellulase activity was 6.5 (34.498 U). The cellulase activity was minimum at pH of 4.5. The factors influencing cellulase activity were carbon sources, pH, temperature, incubation period. This study highlights that CMC as a source of carbon was ideal for exhibiting maximum cellulase activity when compared with other carbon sources. CMC plays a significant positive impact on cellulase production by cellulolytic microbe (Deka *et al.,* 2011). The optimum pH for the production of cellulase by YES5 was 6.5.Cellulase activity is dependent on temperature and pH (Tai *et al.,* 2004). The pH of the medium has a strong influence on the enzymatic processes and the mobility of components across the cell membrane (Moon and Parulekar, 1991).The most popular design employed for optimization of cellulose prduction is Response Surface Methodologies (Queen *et al.,* 2002). RSM offers statistical predictions and actual observation (Singh and Kaur, 2012). For optimising the medium composition for cellulase activity, a natural source of carbon (powdered Napier biomass) was substituted in the place of synthetic carbon source. The combination which the maximum cellulase activity was observed using a natural source of carbon was observed (Table2). The second-order polynomial equation for cellulase activity is as follows:

$$Y = +23.87 + 1.26A + 0.8132B + 0.0277C - 0.1295D - 0.0195E - 0.0135F + 0.3186AB - 0.0902AC + 0.0392AD - 0.0083AE + 0.0705AF + 0.0727BC - 0.0255BD + 0.1589BE - 0.1317BF + 0.1208CD - 0.0548CE - 0.0461CF - 0.0192DE - 0.0817DF + 0.0258EF - 3.10A^2 - 1.99B^2 - 0.5386C^2 - 0.7591D^2 - 0.4366E^2 - 0.1426F^2$$

where Y is the cellulase activity (U/ml) and A, B, C, D, E and F are the test variables. The ANOVA results convey that the model was highly significant and it was obvious with F-value as 48.06 and < 0.0001 p-value. The model showed insignificant lack of fit (19.14). The F, P and lack of fit values indicate that the conducted experiments were in good fit. The predicted R^2 0.9046 was par with the Adjusted R^2 0.9429. The R^2 value was 0.9629, so the model could explain 96 % of total variation. Pure error was 0.0025, which indicated the good reproducibility of the experiments. 3D plots were obtained, which conveyed the best combination of parameters where the cellulase activity was optimum (Fig. 4).

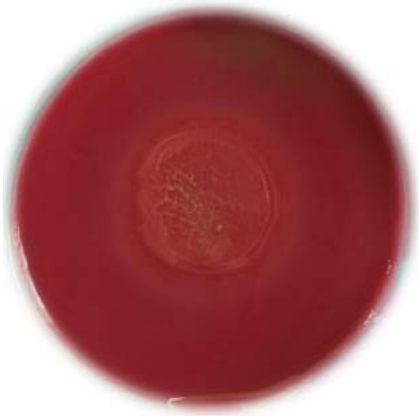

Fig. 1: *Trichosporon asahii* YES5 showing cellulolytic activity. The zone around YES5 colony indicates cellulose hydrolyzing nature.

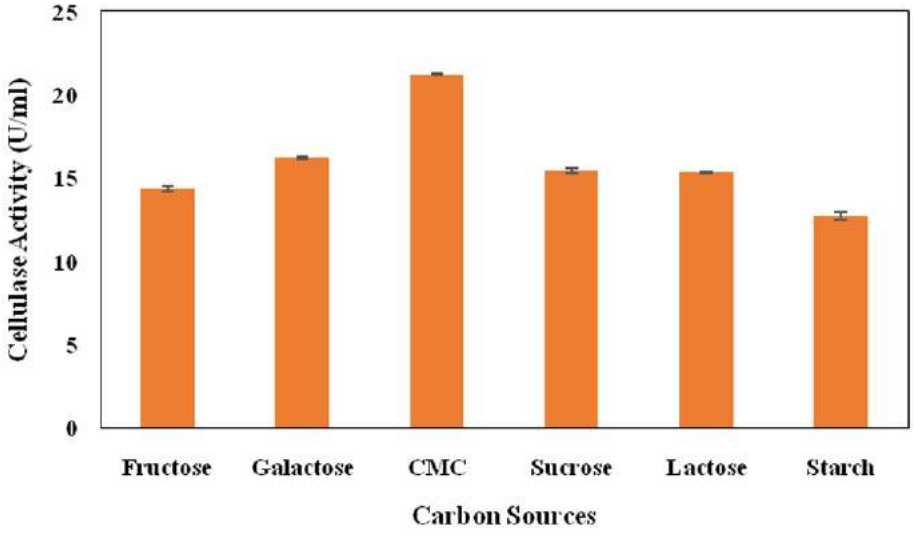

Fig. 2: Effect of carbon sources on cellulase activity of yeast isolate *Trichosporon asahii* YES5. The yeast isolate YES5 was cultured in different mono, di and polysaccharide sources and cellulase assay was done in triplicate. The values are depicted along with standard deviation.

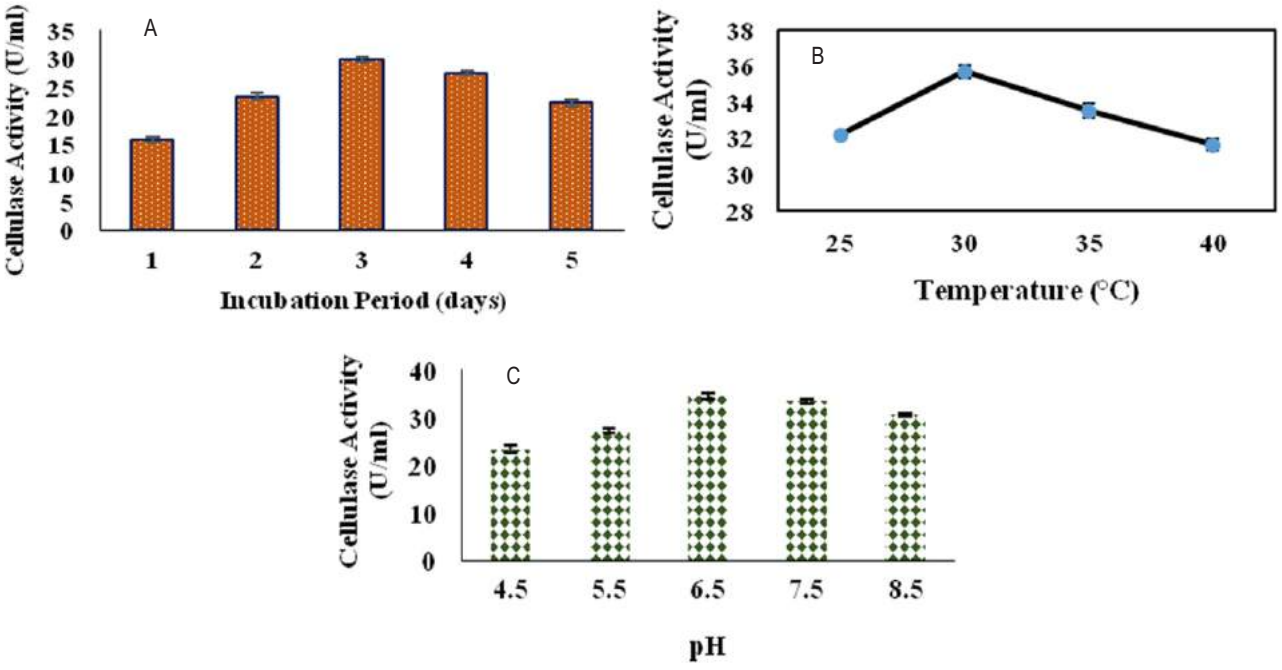

Fig. 3: The effect of process parameters on cellulase assay of *Trichosporon asahii* YES5: (A) Incubation period, (B) Temperature and (C) pH. The assay values was done in triplicate and presented along with standard deviation.

The maximum cellulase activity predicted by the model was observed in the treatment holding the combination of 10g of Napier fodder biomass, 0.2 g K_2HPO_4 , 0.3g $MgSO_4$, 0.217g NaCl, 10g peptone, 0.20g of $(NH_4)_2SO_4$ in 1l of distilled water. The decrease in enzymatic activity with increasing incubation time might be due to depletion of nutrients and production of other inhibitors during the fermentation medium (Ikram-ul-Haq *et al.,* 2005). The optimized parameters were validated practically by shake-flask experiments. The observed cellulase activity for optimized media parameters was 23.87 U ml^{-1}, which is at par with the predicted cellulase activity of 23.55 U ml^{-1}. The results positively correlate that the Napier fodder biomass waste could be utilized by yeast YES5, as a good source of carbon for exhibiting maximum cellulase activity. Cellulase activity using a synthetic source of carbon was 35.70U ml^{-1} at optimal parameters. The cellulase activity using a natural source of carbon (waste Napier biomass) was 23.87 U ml^{-1} at optimal parameters (Table 3). Statistical designs are efficient tool for enhancing cellulase production. This study focused on the optimization of medium components for obtaining maximum cellulase activity in *Trichosporon asahii*, while utilizing Napier biomass waste as a carbon source. The optimum cellulase activity of YES3 (*Candida tropicalis*) isolate, observed after optimizing factors such as pH, temperature and incubation period was 35.53 U (Valliammai *et al.,* 2020). RSM provided the individual and interactive effects of components in the medium during fermentation. Peptone, ammonium chloride, yeast extract were significant components for cellulase production using *Bacillus tequilensi* (Sharma *et al.,* 2015).

Napier biomass was subjected to physico-chemical characterization. The cellulose content was maximum in Napier fodder biomass (51.34 %). The lignin and hemicellulose content of Napier biomass were 19.10 % and 16.70 %, respectively. YES5 was inoculated for the saccharification of Napier biomass waste, and its percentage of saccharification was noted at regular intervals. The samples were withdrawn at regular intervals for estimating the saccharification percentage. The maximum saccharification percentage (33.15%) was observed on 3rd day. Initially, the percentage of saccharification was low followed by a gradual increase, and later a declining trend was noticed (Fig. 5). Yeasts like *Pichiastipitis* was utilized in the saccharification study of sugarcane bagasse (Lau *et al.,* 2010). The saccharification potential of *Candida tropicalis* isolate YES3 in Napier grass biomass was maximum on 3rd day (31.93%) (Valliammai *et al.,* 2020).

In this study, a novel cellulolytic yeast YES5 isolated from the the forest soil was molecularly characterized as Trichosporon asahii (Mk640632). A highly reliable RSM model was developed for maximizing the YES5 cellulase activity. This study contributes an active participant YES5 to the lignocellulosic degrading family which could be a boon for the biobased industries and for the production of green chemicals like ethanol.

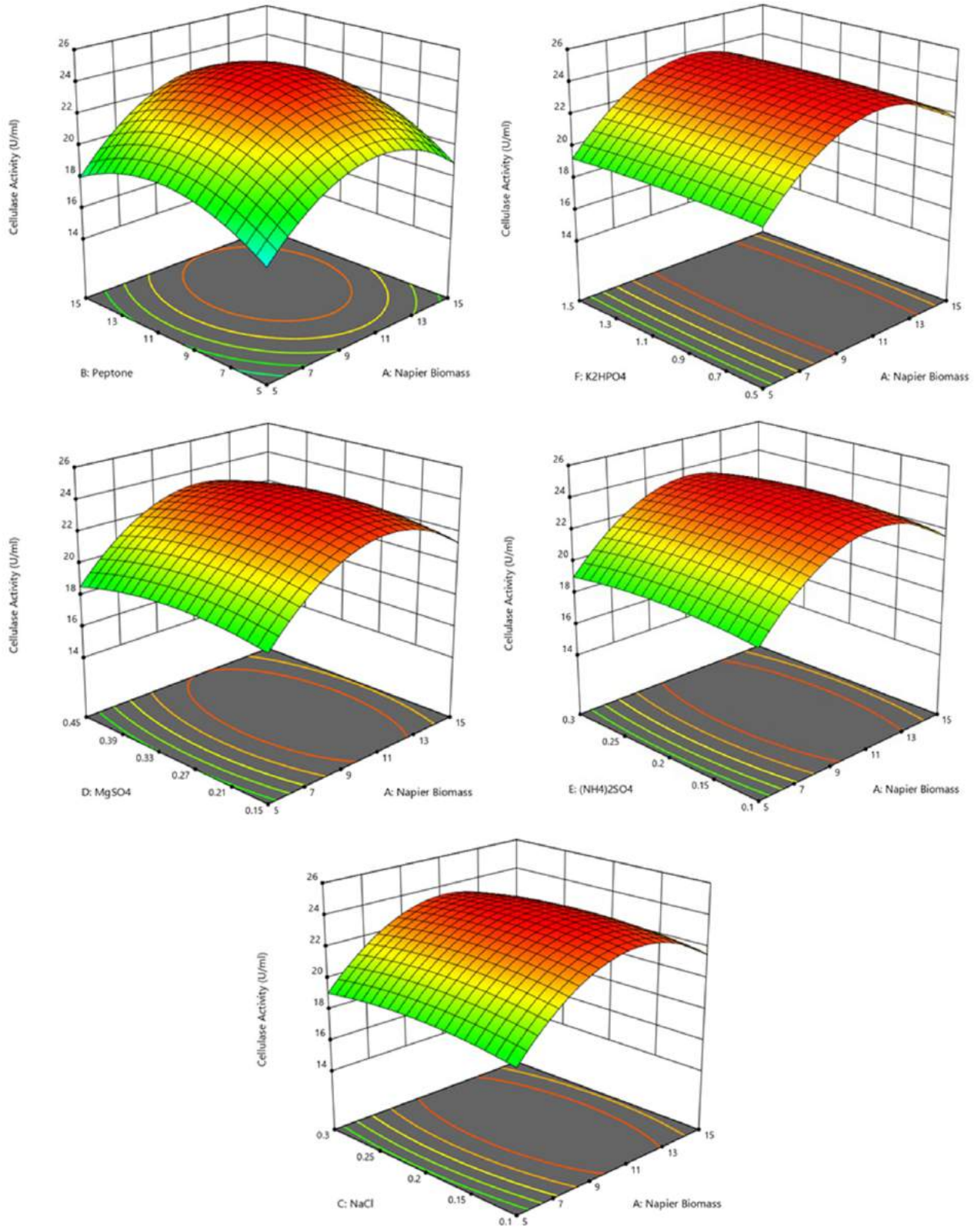

Fig. 4: RSM 3 D plots show the effect of interactive and individual effects of parameters in cellulase activity of *Trichosporon asahii* YES5. (A) Effect of Napier biomass and peptone, (B) Effect of Napier biomass and dipotassium hydrogen phosphate, (C) Effect of Napier biomass and magnesium sulphate, (D) Effect of Napier biomass and Ammonium sulphate and (E) Effect of Napier biomass and sodium chloride.

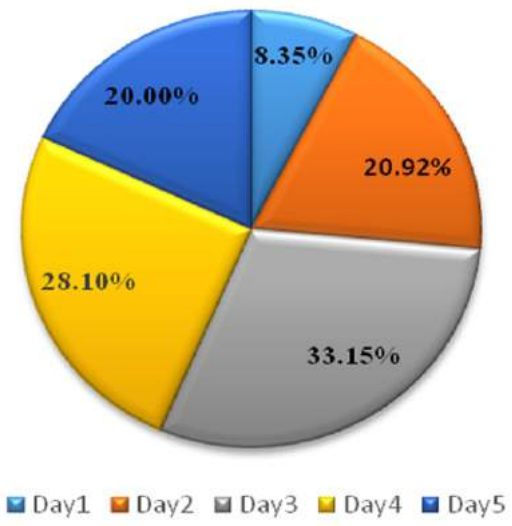

Fig. 5: Saccharification of Napier fodder biomass by *Trichosporon asahii* YES5. YES5 (*Trichosporon asahii*) was inoculated into Napier biomass and saccharification potential was observed for 5 days.

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Add-on Information

Authors' contribution: M.G. Valliammai: Performed scientific experiments and drafted the manuscript; **N.O. Gopal:** Planned the scientific work. He guided Meyyappan Geetha Valliammai in performing the experiments; **R. Anandham:** Suggested ideas for performing the scientific experiment.

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Host-specific influence on early growth and physiological attributes of sandal (*Santalum album*) grown in farmlands

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Abstract

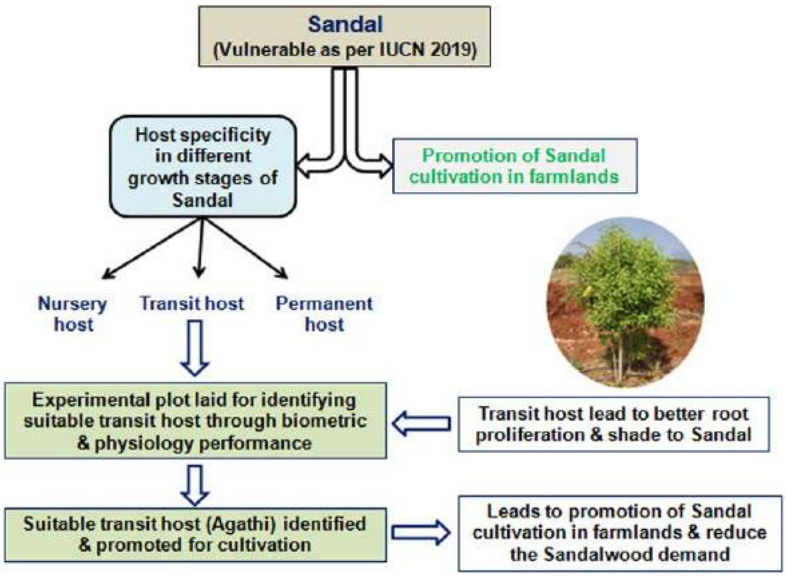
Aim: The present study aimed to investigate the host- specific compatibility of sandal (*Santalum album*) for better root proliferation and boosting the growth of tree.

Methodology: Sandal (*Santalum album*) was grown with 8 different leguminous and non-leguminous species at early stage to evaluate its biometric growth (height and basal diameter) and physiological (chlorophyll, photosynthesis rate and transpiration rate) performance under field conditions.

Results: Sandal grown with *Sesbania grandiflora* recorded highest biometric observations, chlorophyll, photosynthesis and transpiration rate followed by Sandal + *Albizia lebbeck* and sandal + *Casuarina junghuhniana*. The sandal grown with leguminous host showed superior growth performance than sandal grown with non-leguminous host.

Interpretation: The present study recommended that *Sesbania grandiflora* can be grown as transit host for boosting the growth of sandal.

Key words: Hemi-root parasites, Host-specific compatibility, Root proliferation, Sandal



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Introduction

Globally, tropical forests cover merely 7% of Earth's land surface and harbour more than half of the world's species (Wilson, 1988) which are highly threatened by human activities (Htun *et al.,* 2011). Researchers have predicted that clearing half of the world's residual forests would remove 85 % of all the species inhabited by the people (Pimm and Raven, 2000; Pandey *et al.,* 2018). Data from tropical forest suggested that, continuous loss of more than one higher plant species per day (Myers, 1990), disappearance of 20 ha forests, destruction of more than 1800 population per hour (Hughes *et al*., 1997) and loss of species population at a percentage rate of 3-8 times than the rate of species extinction due to natural disturbance, biotic disturbance and habitat alterations (Costanza *et al.,* 1997; Kittur *et al.,* 2014; Baboo *et al.,* 2017; Maurya *et al.,* 2019). In India, tropical forest covers about 80.69% of the total forest land and tropical dry deciduous forest accounts for 41.87 %of total forest (FSI 2011). These anthropogenic disturbances have lead to land degradation, species replacement and species disappearance from their natural habitat (Bargali *et al.,* 2018; Bargali *et al.,* 2019; Manral *et al.,* 2020).

Parasitism is the concept of deriving some part or all of the water and nutrition from other living trees. In every biome, about 1 per cent of the plants/trees belong to angiosperms with the character of parasitism. The nature of parasitic plants is formation of modified root called haustoria, which penetrate into the host plant and joins the conductive system of xylem, phloem or both. Among different angiosperms tree species, sandal (*Santalum album*) is a valuable tree species with semi-root parasite nature with root penetration into host plant (Radomiljac *et al.,* 1998, Yang *et al.,* 2014; Marshall *et al.,* 1994). Sandal trees partially rely on the host plant to take up water and nutrient through the functional haustoria eventhough sandal trees have functional chloroplast to perform photosynthesis. Indian sandalwood is superior in world market for its aromatic heartwood and root (Kim *et al.,* 2005), however, due to the illicit felling and smuggling in the past, the sandal population in the country has declined and reached to the vulnerable status (IUCN, 2020). Due to the increasing anthropogenic pressure, the declining population of sandalwood in the country has lead to the production variation of 4000 Mg during 1950s to 500 Mg in 2007 from. The natural population of sandal in India was recorded mainly in Deccan plateau (Karnataka, Tamil Nadu, Kerala and Andhra Pradesh) on peninsular region (Gairola *et al.,* 2008).

To overcome this scenario, cultivation of sandal in the farmlands have been promoted in the countries like India, Australia, China and Indonesia (Dhanya *et al.,* 2010) through farm forestry, agroforestry, home gardens (Balasubramanian *et al.,* 2018). The poor understanding of parasite-host relationship leads to problematic of both during seed germination, regeneration and establishment of sandal (Surendran *et al.,* 1998). During the life cycle of sandal, total three host are recommended for its better establishment, growth and function. Three hosts, *viz.,* nursery host are planted during germination of sandal seedlings to facilitate the uptake of nutrients through prolific rooting of host (Ehrhart and Fox, 1995); Transit host are planted to bride the stage of field establishment at early stage of growth up to 2 years through interim support for sandal root system, partial shading and also enhanced micro-climate and permanent/ long term host planted after the transit host and the host leads to prolonged support through permanent root association, formation of functional haustorial network and enhance uptake of nutrients. Promotion of sandal with host throughout the life cycle will built up the growth, but, once the host was removed wilting and leaf shedding on sandal tree was recorded (Roche *et al.,* 2014). Still there is unanimity of accepting the hemi-parasitic nature of sandal, as host playing a role in supplying water and nutrient to the sandal. Interestingly, many of the research studies have proven the successful establishment of sandal and host plant interaction in nursery and field condition. Considering all these factors, a field level experiment was carried out to identify the host suitability for transit host (Intermediate host) in sandal by studying the growth and physiological parameters.

Materials and Methods

The study was carried out in one-year-old Sandal plantation established and maintained at Forest College and Research Institute, Mettupalayam, Coimbatore, India. The soil of the Sandal plantation established with the host was Illupanatham soil series, slightly alkaline (pH-7.87) in nature; soil was loamy sand, well drained and non saline (EC-0.20 dSm^{-1}).

Experimental design: To identify the host suitability sandal during transit host (Intermediate host), the host species selected are *Cynodon dactylon*, *Chrysopogon zizanioides, Vigna unguiculata, Tephrosia purpurea, Sesbania grandiflora, Vachellia nilotica, Albizia lebbeck* and *Casuarina junghuhniana*. Totally nine treatments, *viz.,* T_1: Sandal + *Cynodon dactylon*; T_2: Sandal + *Chrysopogon zizanioides*; T_3: Sandal + *Vigna unguiculata*; T_4: Sandal + *Tephrosia purpurea*; T_5: Sandal + *Sesbania grandiflora*; T_6: Sandal + *Vachellia nilotica*; T_7: Sandal + *Albizia lebbeck*; T_8: Sandal + *Casuarina junghuhniana* and T_9: Control (Sandal alone) with three replication in a randomized block design. The host suitability study of sandal during field establishment was initated with drip irrigation and irrigation schedule was once in every three days during summer/non rainy days for first six months and at later stages it was irrigated for twice/week with the discharge rate of 4.0 $l\,hr^{-1}$ for one hour/day.

Biometric calculation: Biometric characteristics *viz.,* height and basal diameter were measured during 6 months after planting (MAP), 9 MAP and 12 MAP. The height of the trees was measured from the ground level to the leading terminal tip using standard scale and was expressed in meter. Basal diameter is measured with the help of digital vernier caliper at the ground level and expressed in centimeters.

Chlorophyll content: The concentrations of chlorophyll 'a', chlorophyll 'b', total chlorophyll and chlorophyll a/b ratio were

estimated by the method of Yoshida *et al.* (1976) and expressed as mg g^{-1} f.wt. Fully matured young fresh leaf samples (250 mg) were collected, washed in distilled water and then ground in 80% acetone using pestle and mortar. The homogenate solution was centrifuged at 500 rpm for 10 min. The supernatant was collected and the volume was made up to 25 ml with 80% acetone. The optical density of the content was measured at 663 and 645 nm using double beam UV Spectrophotometer. The chlorophyll 'a', chlorophyll 'b' and total chlorophyll content were calculated.

Estimation of leaf photosynthesis and transpiration rate: Using a Portable Photosynthesis System (PPS, model LCpro + Photosynthesis System CO_2 gas analyzer, UK), the net rate of photosynthesis rate and transpiration rate were estimated for one-year- sandal plantation with the different host between 09.00 am to 11.00 am for three sunny days for effective results. The observed CO_2 concentration during field experiment varied between 350 ppm to 360 ppm with the leaf temperature of 32.5°C on fully matured leaves (5-6 leaves from the bud).

Statistical analysis: Data were subjected for statistical analysis to evaluate the possible relationship between different parameters and to employ analysis of variance (Gomez and Gomez, 1984). The comparison between different host planted in sandal were assessed using ANOVA and the physiological analysis were carried out through SPSS.

Results and Discussion

Sandal plants showed a significant growth performance in terms of (height and basal diameter) in association with host species, when compared with Control (Sandal alone). This clearly indicated that the host-tree interaction in sandal had influenced the growth. Among the eight different host planted, the height (1.96) and basal diameter (4.39) cm were significantly greater in *Sesbania grandiflora* with during one-year-of growth (Table 1 and Figure 1). In a similar study Balasubramanian *et al.* (2018) stated that sandal growth with *Alternanthera sessilis* + *Sesbania grandiflora* recorded superior growth with height of 1.45 m during 8 months after planting. Sandal grown with leguminous host showed superior growth performance than sandal grown with non-leguminous host (Durairaj and Kamaraj, 2016).

The xylem tapping root Hemiparasite was grown superior during the association with nitrogen-fixing hosts (Lu *et al.,* 2014; Press and Phoneix, 2005; Bell and Adams, 2011). On competing next to *Sesbania grandiflora*, Sandal showed good biometric growth in *Albizia lebbeck* and *Casuarina junghuhniana* with height of 1.77 m and 1.71 m. The lowest growth performance (Height- 1.02 m and Basal diameter- 2.22 cm) was observed in control (Sandal alone). In order to support the present investigation, Durairaj and Kamaraj (2016) exhibited that growth increment was observed in sandal grown with host than sandal without host. Host-sandal interaction is related to uptake, translocation of various mineral nutrients and photosynthesis efficiency (Ananthapadmanabha *et al.,* 1984; Rangaswamy *et al.,* 1986; Brand 2002; Lu *et al.,* 2014; Rocha *et al.,* 2014; Balasubramanian *et al.,* 2018). In the present study, the maximum chlorophyll content was recorded in Sandal + *Sesbania grandiflora,* followed by Sandal + *Casuarina junghuhniana* (Table 2). Eventhough sandal have functional chloroplast to perform photosynthesis, the functional haustoria duly played an important role in water uptake and physiological action of the parasite (Pate,

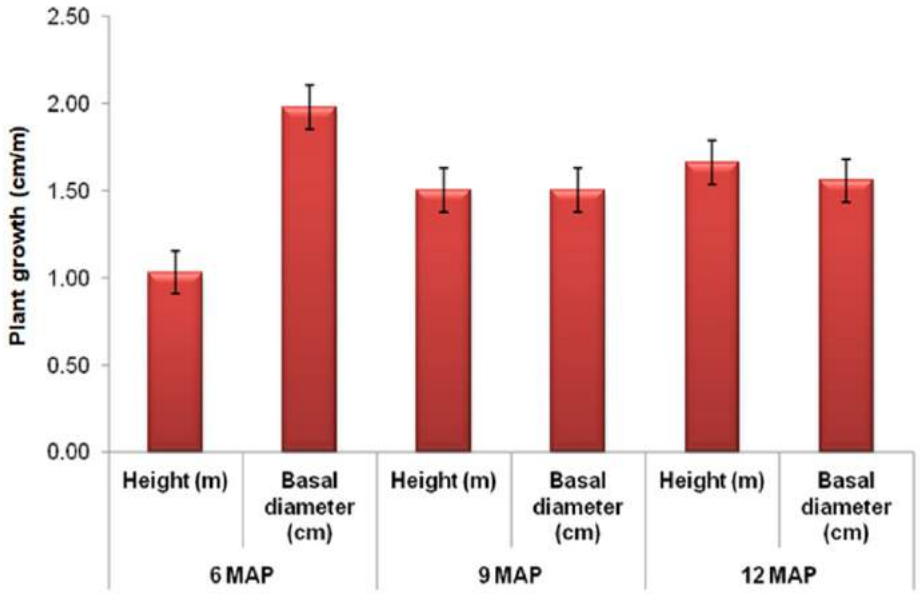

Fig. 1: Influence of different host association on growth performance of sandal (*Santalum album*) at different stages.

Table 1: Host-specific interactions on height and basal diameter of sandal

	Treatments	6 MAP		9 MAP		12 MAP	
		Height (m)	Basal diameter (cm)	Height (m)	Basal diameter (cm)	Height (m)	Basal diameter (cm)
T_1	Sandal + *Cynodon dactylon*	0.99	1.87	1.21	2.24	1.58	2.96
T_2	Sandal + *Chrysopogon zizanioides*	1.04	2.05	1.29	2.78	1.66	2.46
T_3	Sandal + *Vigna unguiculata*	1.03	2.23	1.30	2.86	1.65	1.98
T_4	Sandal + *Tephrosia purpurea*	0.98	1.90	1.19	2.55	1.46	2.16
T_5	Sandal + *Sesbania grandiflora*	1.21	2.41	1.45	3.25	1.96	4.39
T_6	Sandal + *Vachellia nilotica*	1.08	1.99	1.31	2.63	1.65	3.62
T_7	Sandal + *Albizia lebbeck*	1.18	2.27	1.34	3.01	1.77	3.29
T_8	Sandal + *Casuarina junghuhniana*	1.07	1.98	1.17	2.76	1.71	3.89
T_9	Control	0.69	1.12	0.91	1.99	1.02	2.22
	S.Ed	0.031	0.070	0.046	0.118	0.070	0.162
	CD (0.05)	0.065	0.147	0.096	0.248	0.148	0.341

Table 2: Influence of different host association on chlorophyll content (mg g^{-1}) of one-year-old sandal

	Treatments	Chlorophyll 'a'	Chlorophyll 'b'	Total chlorophyll	Chlorophyll a/b ratio
T_1	Sandal + *Cynodon dactylon*	0.548	0.328	0.876	1.671
T_2	Sandal + *Chrysopogon zizanioides*	0.606	0.291	0.897	2.082
T_3	Sandal + *Vigna unguiculata*	0.698	0.363	1.061	1.923
T_4	Sandal + *Tephrosia purpurea*	0.611	0.289	0.900	2.114
T_5	Sandal + *Sesbania grandiflora*	0.790	0.470	1.260	1.681
T_6	Sandal + *Vachellia nilotica*	0.601	0.300	0.901	2.003
T_7	Sandal + *Albizia lebbeck*	0.721	0.317	1.038	2.274
T_8	Sandal + *Casuarina junghuhniana*	0.700	0.398	1.098	1.759
T_9	Control	0.500	0.195	0.695	2.564
	S.Ed	0.026	0.013	0.047	0.099
	CD (0.05)	0.056	0.027	0.099	0.209

Table 3: Influence of different host association on photosynthesis rate and transpiration rate on one-year-old sandal

	Treatments	Photosynthesis rate (μ mol. m^{-2} s^{-1})	Transpiration rate (m mol. m^{-2} s^{-1})
T_1	Sandal + *Cynodon dactylon*	12.75	4.67
T_2	Sandal + *Chrysopogon zizanioides*	13.23	5.02
T_3	Sandal + *Vigna unguiculata*	12.71	4.22
T_4	Sandal + *Tephrosia purpurea*	8.76	3.87
T_5	Sandal + *Sesbania grandiflora*	18.42	5.56
T_6	Sandal + *Vachellia nilotica*	10.11	3.90
T_7	Sandal + *Albizia lebbeck*	15.55	5.33
T_8	Sandal + *Casuarina junghuhniana*	15.49	5.08
T_9	Control	7.56	3.73
	S.Ed	0.586	0.232
	CD (0.05)	1.233	0.487

T_1: Sandal + *Cynodon dactylon*; T_2: Sandal + *Chrysopogon zizanioides*; T_3: Sandal + *Vigna unguiculata*; T_4: Sandal + *Tephrosia purpurea*; T_5: Sandal + *Sesbania grandiflora*; T_6: Sandal + *Vachellia nilotica*; T_7: Sandal + *Albizia lebbeck*; T_8: Sandal + *Casuarina junghuhniana* and T_9: Control

2001; Bell and Adams, 2011; Cameron and Seel, 2007).

Sandal plants grown with the host showed significant increase in rate of photosynthesis and transpiration than without host, especially sandal with leguminous host showed an increasing trend. An interesting finding from this study was that the Sandal grown with *Sesbania grandiflora* recorded highest photosynthesis rate of 18.42 µ mol. m^{-2} s^{-1} and transpiration rate of 5.56 m mol. m^{-2} s^{-1} followed by Sandal + *Albizia lebbeck* and Sandal + *Casuarina junghuhniana* with photosynthetic rate of 15.55 µ mol. m^{-2} s^{-1} and 15.49 µ mol. m^{-2} s^{-1}. The photosynthetic rate of leguminous tree species Albizia lebbeck is on par with the non-leguminous tree species *Casuarina junghuhniana* (Table 3). Lu *et al.* (2014) found that improved rates of photosynthesis in *Santalum album*, when grown in association with N_2 fixing *Acacia confusa* and *Dalbergia odorifera* provide circumstantial evidence that *Santalum album* has a lower dependence on such hosts for carbon compared with non nitrogen fixing *Bischofia polycarpa* and *Dracontomelon duperreranum*.

In the present study, sandal with host showed superior photosynthetic rate (carbon assimilation rate) than sandal grown without host, which is in on par with the study conducted by Rocha *et al.* (2014). The photosynthesis rate of 7.56 and transpiration rate of 3.73 was recorded in Sandal without host in the present study. A significant difference was observed in rate of photosynthesis between sandal with host and without host (Tennakoon *et al.,* 1997). Jiang *et al.* (2003) and Taylor *et al.* (1996) indicated that *Santalum album* clearly appears to optimize root xylem sap extraction from its hosts in the same way as obligate hemiparasite *Striga hermonthica* and facultative hemiparasite *Rhinanthus minor* by having higher transpiration rates and lower Water Use Efficiency (WUE) values than in the host.

In conclusion, sandal along with *Sesbania grandiflora* showed better growth and physiological performance at early stage as transit host. *Sesbania grandiflora* performed better as transit host to sandal upto 2 years by inducing good root proliferation and excellent haustoria association, which help in water translocation and uptake of various mineral nutrients. The complex interaction between parasite and host (*Sesbania grandiflora*) during early growth stage of *Santalum album*, boosted the growth during initial stage of plantation.

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Add-on Information

Authors' contribution: A. Balasubramanian: Field preparation as per different sandal treatments and corrected the paper; **C.N. Hari Prasath**: Collected biometric data and ecophysiological data. Formulated the data; **S. Radhakrishnan:** Statistical analysis of data; **M. Sivaprakash:** Collection of literature related to sandal.

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Studies on population dynamics of major pests of *Ailanthus excelsa* Roxb

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Abstract

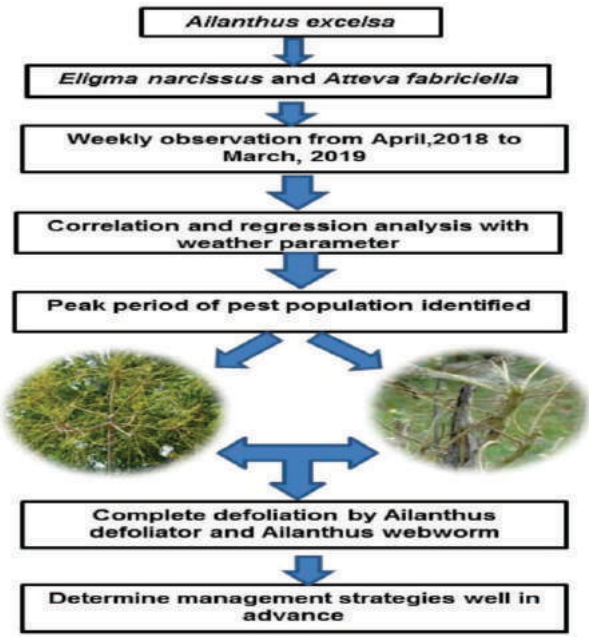
Aim: To study the peak period of incidence of major pest of *Ailanthus excelsa*.

Methodology: Seasonal abundance of major insect pests of *A. excelsa viz.,* ailanthus defoliator and ailanthus webworm were studied in 5-year-old plantation at Forest College and Research Institute, Mettupalayam from April, 2018 to March, 2019. Weekly observations were made on the abundance of major insect and pest population which were correlated with weekly weather parameters.

Results: Monitoring the population dynamics of insect pests revealed that the major key pests were ailanthus defoliator and webworm. The highest number of ailanthus defoliator (38.00 larvae per tree) and webworm (33.90 larvae per tree) were recorded during 44th standard week in 5-year-old plantation. Correlation analysis revealed that maximum temperature (T_{max}) was negatively correlated with ailanthus defoliator with the r value of -0.299. Regarding webworm, wind velocity and evaporation rate were negatively correlated with the r value of -0.412 and -0.361 and was found to be statistically significant at 1% level.

Interpretation: Seasonal abundance of this information helps us to take decision for the management of *A. excelsa pests.*

Key words: *Ailanthus excelsa*, Defoliator, Population dynamics, webworm



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Introduction

Ailanthus excelsa is a multipurpose, deciduous, fast growing tree species belonging to family Simaroubaceae. It grows well in arid and semi-arid regions, and is suitable for planting in dry areas experiencing annual rainfall of 400 mm. In India, the tree is indigenous to southern and central India and also distributed in Western Peninsula, Rajasthan, Bihar, Orissa, Bundelkhand in Madhya Pradesh, Broach and Panchamal districts of Gujarat, in dry deciduous forests of Maharashtra and scarce in Deccan and Karnataka. It avoids moist areas having high rainfall. It grows in broad range of soil types including sandy soils, adapts to drought stress and can tolerate dry season of 4 to 6 months (Rajasugunasekar, 2014).The demand for *A. excelsa* is increasing due to its multipurpose uses *viz.,* leaves as fodder and stem in the production of match wood, box plank, packing cases, sword sheath, paper, toys, plywood veneers (Sharma *et al*., 2016), pencil (Lavhale and Mishra, 2007) and fuel wood (Jat *et al*., 2011). Among the various pests, *Eligma narcissus* and *Atteva fabriciella* are the major pests of *Ailanthus excelsa*. *Eligma narcissus* causes complete defoliation of seedlings in the nursery and up to 95% defoliation in the young plantations up to 5-years-old, resulting in loss of growth increment (Varma, 1996). Defoliation by *Atteva fabriciella* leads to reduction in growth increment due to defoliation of tender leaves, forking of tree due to terminal bud damage and loss of seed production due to damage of inflorescence and fruits (Varma, 1996; Nair, 2007).

Wolda, (1988); Pinhiero *et al*. (2002) reported that the common phenomenon for the abundance of tropical insect is seasonal variation. Change in insect abundance occurs over time for various reasons such as micro and macro climate change and variation in the availability of food resources. Insect can operate faster and more efficiently at higher temperature and can also feed, develop, reproduce and disperse once the climate is warm, although they live for a short period (Drake, 1994). Information on weather parameters of standard weeks is important compared to the annual mean to analyze the population dynamics of pests and natural enemies in relation to changing environmental conditions. Understanding this impact of native weather variables on insect population dynamic is vital for managing pests of tropical crops (Lomelí-Flores *et al.,* 2010)

Therefore, this study was undertaken to determine the effect of climatic parameters on the seasonal abundance of insect pests of *A. excelsa.*

Materials and Methods

Seasonal abundance of major insect pests of *A. excelsa viz.,* Ailanthus defoliator and Ailanthus webworm were studied in 5-year-old plantation at Forest College and Research Institute, Mettupalayam from April, 2018 to March, 2019. Weekly observation were made on the abundance of major insect pests on 10 randomly selected trees on three leaves in the lower whorls for ailanthus defoliator and three leaves on the crown for ailanthus webworm. Weekly counts of insect pest population was correlated with weekly weather parameters *viz.,* maximum temperature (T_{max}), minimum temperature (T_{min}), relative humidity (RH), rainfall, sunshine, wind speed and evaporation rate obtained from Agro-meteorological Observatory at Forest College and Research Institute, Mettupalayam.

Correlation and multiple linear regression analyses were conducted to assess the relationship between seasonal abundance of insect pests in the field and weather parameters *viz.,* maximum temperature (T_{max}), minimum temperature (T_{min}), relative humidity (RH), rainfall, sunshine, wind speed and evaporation rate were carried out using SPSS statistics ver.17.0. (Gomez and Gomez, 1984).

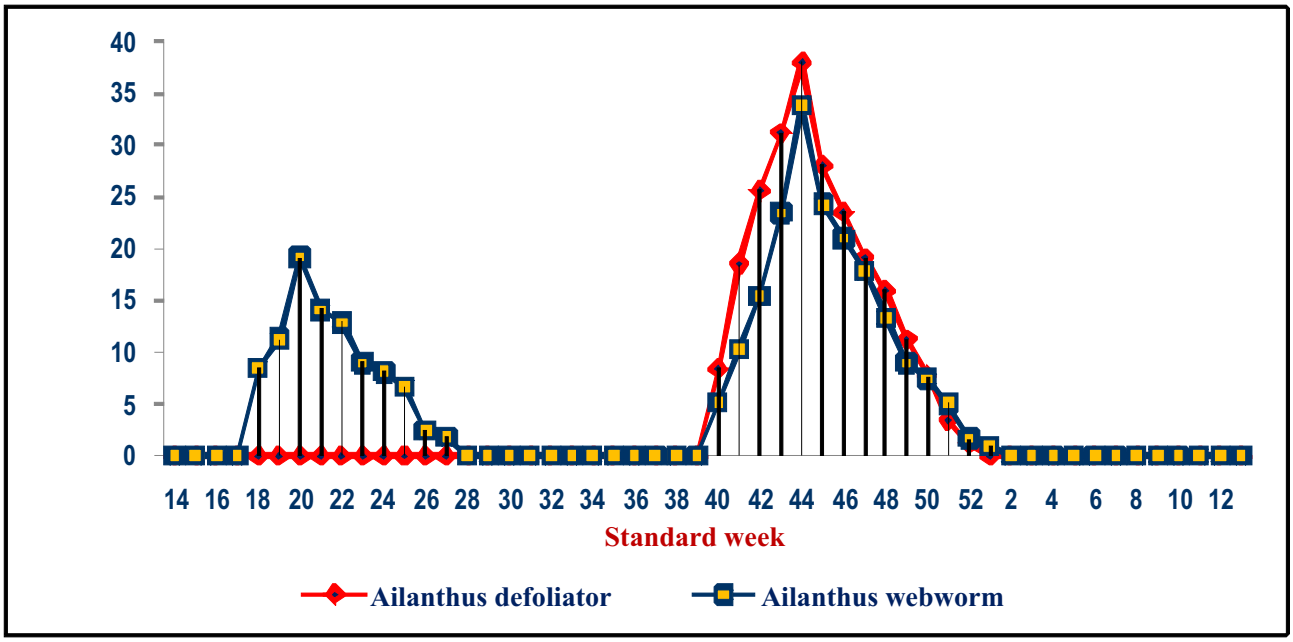

Fig.1.: Seasonal abundance of defoliators infesting 5-year-old plantation of *Ailanthus excelsa* during 2018-2019

Table 1: Monitoring insect pests in 5-year-old plantation of *A. excelsa* during 2018-19*

Date / Months	Standard week	Ailanthus defoliator (No. per tree)	Ailanthus webworm (No. per tree)	Defoliation (%)
2018				
01 to 07 April	14	0.00	0.00	0.00
08 to 14 April	15	0.00	0.00	0.00
15 to 21 April	16	0.00	0.00	0.00
22 to 28 April	17	0.00	0.00	0.00
30 to 06 May	18	0.00	8.50	1.00
07 to 13 May	19	0.00	11.30	5.00
14 to 20 May	20	0.00	19.20	8.00
21 to 27 May	21	0.00	14.10	12.00
28 to 03 June	22	0.00	12.90	15.00
04 to 10 June	23	0.00	9.00	10.00
11 to 17 June	24	0.00	8.10	8.00
18 to 24 June	25	0.00	6.70	5.00
25 to 01 July	26	0.00	2.40	2.00
02 to 08 July	27	0.00	1.80	0.00
09 to 15 July	28	0.00	0.00	0.00
16 to 22 July	29	0.00	0.00	0.00
23 to 29 July	30	0.00	0.00	0.00
30 to 05 August	31	0.00	0.00	0.00
06 to 12 August	32	0.00	0.00	0.00
13 to 19 August	33	0.00	0.00	0.00
20 to 26 August	34	0.00	0.00	0.00
27 to 02 September	35	0.00	0.00	0.00
03 to 09 September	36	0.00	0.00	0.00
10 to 16 September	37	0.00	0.00	0.00
17 to 23 September	38	0.00	0.00	0.00
24 to 30 September	39	0.00	0.00	0.00
01 to 07 October	40	8.40	5.20	2.00
08 to 14 October	41	18.60	10.30	25.00
15 to 21 October	42	25.60	15.40	35.00
22 to 28 October	43	31.20	23.50	55.00
29 to 04 November	44	38.00	33.90	75.00
05 to 11 November	45	28.00	24.30	80.00
12 to 18 November	46	23.50	21.00	82.00
19 to 25 November	47	19.10	17.80	85.00
26 to 02 December	48	16.00	13.40	88.00
03 to 09 December	49	11.30	9.00	70.00
10 to 16 December	50	7.70	7.50	55.00
17 to 23 December	51	3.50	5.10	30.00
24 to 31 December	52	1.20	1.70	10.00
2019				
01 to 07 January	01	0.00	0.90	3.00
08 to 14 January	02	0.00	0.00	0.00
15 to 21 January	03	0.00	0.00	0.00
22 to 28 January	04	0.00	0.00	0.00
29 to 04 February	05	0.00	0.00	0.00
05 to 11 February	06	0.00	0.00	0.00
12 to 18 February	07	0.00	0.00	0.00
19 to 25 February	08	0.00	0.00	0.00
26 to 04 March	09	0.00	0.00	0.00
05 to 11 March	10	0.00	0.00	0.00
12 to 18 March	11	0.00	0.00	0.00
19 to 25 March	12	0.00	0.00	0.00
26 to 01 April	13	0.00	0.00	0.00
Mean	**-**	**4.46**	**5.44**	**14.63**

* - Data presented without statistical analysis

Results and Discussion

Monitoring of seasonal abundance of insect pests in five years old plantation by direct counting revealed that the highest number of ailanthus defoliator was recorded during 44th standard week of 2018 (38.00 larvae per tree) and the minimum number of larvae was recorded during 52nd standard week (1.20). Ailanthus defoliator was not noticed till 39th standard week of 2018 in 5-year-old plantation. The incidence started during 40th standard week (8.40 larvae per tree), increased gradually, attained peak during 44th standard week and then declined thereafter. The larval population disappeared from first to 13th standard week of 2019 (Figure 1) and coincide with the findings of findings of Varma (1986) who has reported the peak incidence of both *E. narcissus* and *A. fabriciella* during September to January in 2-year-old plantation of *A. triphysa* in Kerala.

Regarding Ailanthus webworm, the highest number of webworm was recorded during 44th standard week (33.90 larvae per tree) and the minimum number larvae was recorded during 1st standard week of 2019 (0.90 larvae per tree). During the observation period of one year, the population of webworm was noticed twice. The first incidence started during 18th standard week (8.5 per tree), increased gradually, attained first peak during 20th standard week (19.20), declined gradually and disappeared from 28th to 39th standard week. Second incidence of webworm started during 40th standard week and extended till first standard week of 2019, with the second peak population (33.90 larvae per tree) during 44th standard week (Table 1). The present study are in conformity with the earlier study, where, *A. triphysa* defoliators was found to be abundant from November to February, coinciding with the general flushing period of tree and smaller population was present throughout the year, thriving on small quantities of new leaves (Varma, 1991) and the peak population of *A. fabriciella* during the onset of monsoon in June-July, with the lowest population in summer months (Mathur *et al*.,1970).

The results of correlation analysis between weather parameters and abundance of ailanthus defoliator revealed that the maximum temperature (T_{max}) was negatively correlated with the population of Ailanthus defoliator with the r value of -0.299 and was found to be statistically significant at 5% level. The minimum temperature, relative humidity and sunshine were also negatively correlated with r value of -0.259, -0.143 and -0.364, respectively. Rainfall was positively correlated with r value of 0.206, while, wind velocity and evaporation rate were negatively correlated with r value of -0.495 and -0.499, respectively and were found to be statistically significant at 1% level (Table 2). The

Table 2. Influence of weather parameters on seasonal abundance of Ailanthus defoliator and A. webworm in 5-year-old plantation during 2018-19

Variables	Correlation coefficient	
	Ailanthus defoliator	Ailanthus webworm
Maximum temperature (T_{max}) (°C)	-0.299**	-0.133
Minimum temperature (T_{min}) (°C)	-0.259	-0.134
Relative humidity (%)	-0.143	-0.222
Rainfall (mm)	0.206	0.163
Sunshine (h/day)	-0.364	-0.091
Wind velocity (km/h)	-0.495*	-0.412*
Evaporation rate (mm)	-0.499*	-0.361*

**Correlation coefficient is significant at 5% level
*Correlation coefficient is significant at 1% level

Table 3. Multiple linear regression analysis for the prediction of seasonal abundance of Ailanthus defoliator and A. webworm in 5-year-old plantation during 2018-19

Variables	Regression coefficient	
	Ailanthus defoliator (Y_1)	Ailanthus webworm (Y_2)
Intercept (a)	20.526	30.103
Maximum temperature (T_{max}) (°C) (X_1)	1.344*	0.555
Minimum temperature (T_{min}) (°C) (X_2)	0.436	0.908**
Relative humidity (RH) (%) (X_3)	-0.394	-0.429*
Rainfall (mm) (X_4)	-0.013	-0.043
Sunshine (h/day) (X_5)	0.035	4.077*
Wind velocity (km/h) (X_6)	-4.178	-3.960**
Evaporation rate (mm) (X_7)	-7.074**	-10.943*
R^2	0.374	0.463

Regression equations
1) Y_1= 20.085 + 1.334*X_1 + 0.436X_2 – 0.394X_3 – 0.013X_4 + 0.035X_5 – 4.178X_6 – 7.074**X_7; 2) Y_2= 30.103 +0.555X_1 + 0.908**X_2 – 0.429*X_3 – 0.043X_4 + 4.077*X_5 – 3.960**X_6 – 10.943*X_7; **Regression coefficient is significant at 5% level
*Regression coefficient is significant at 1% level

present findings are in conformity with the findings of Kumar et al. (2007) who have reported that tobacco caterpillar, *Spodoptera litura* had non-significant correlation with maximum temperature, minimum temperature, relative humidity and rainfall.

In case of Ailanthus webworm incidence, rainfall was positively correlated with webworm population with the r value of 0.163. Whereas, wind velocity and evaporation rate were negatively correlated with the r value of -0.412 and -0.361 and were found to be statistically significant at 1% level. The maximum temperature (T_{max}), minimum temperature (T_{min}), relative humidity and sunshine were negatively correlated with the population of webworm with the r value of -0.133, -0.134, -0.222 and -0.091 (Table 2). The present study is in conformity with the findings of Varma (1986) who reported that occurrence of defoliators was low during rainy season, but there was no definite correlation between pest incidence and rainfall because of population of *A. fabriciella* was observed throughout the year(Varma, 1991) and the population of leaf webber were negatively correlated with minimum and maximum temperature and positively correlated with relative humidity and rainfall in *Vigna mungo* (Umesh *et al.*, 2010).

Multiple linear regression analysis between weather parameters and abundance of Ailanthus defoliator in 5-year-old plantation revealed that evaporation rate had significant contribution towards Ailanthus defoliator population with the R^2 value of 0.374at 5% level. When the rate of evaporation increased by 1 mm, the mean number of larval population decreased by 7.074 per tree. The maximum temperature (T_{max}) also had significant contribution towards the defoliator population at 1% level. When the maximum temperature increased by 1°C, defoliator population increased by 1.344 larvae per tree (Table 3).

The minimum temperature and wind velocity had significant contribution towards the abundance of Ailanthus webworm at 5% level with R^2 value of 0.463. When minimum temperature increased by 1°C, the webworm population increased by 0.908 larvae per tree, whereas, increase in wind velocity by 1 km h^{-1} had decreased the webworm population by 3.960 larvae per tree. Relative humidity, sunshine and evaporation rate also contributed significantly towards the webworm population at 1% level. When the relative humidity increased by 1%, larval population decreased by 0.429 per tree whereas increase in sunshine by 1h/day had significantly increased the population of webworm by 4.077 per tree. When the evaporation rate increased by 1 mm, the webworm population decreased by 10.943 larvae per tree (Table 3). The results of present study on defoliators are in conformity with the finding of William and Andrew (1995) who reported that increase in temperature by 2°C had reduced the defoliation by spruce budworm and increased defoliation by gypsy moth. William and Andrew (1995) has also stated that increase in temperature by 2°C with an increase of 0.5 mm precipitation per day had increased the defoliation by both budworm and gypsy moth in spruce. Guedes *et al.* (2000) reported that 6 to 7 years old plantations of *Eucalyptus grandis* under the temperature of 18° C registered maximum incidence of *Stenalcidia grosica.*

Considering the significance of Ailanthus as a multipurpose agroforestry tree species, this study on seasonal abundance of pests of *Ailanthus excelsa* will be helpful to determine the peak period of pest incidence in Ailanthus and then correlation between the defoliator population and weather parameters will help to predict the pest outbreak, forewarn the farmers and to determine the management strategies well in advance.

Add-on Information

Authors' contribution: V. Manimaran: Writing full article and statistical analysis; **M. Suganthy:** Technical guidance for table preparation; **A. Balasubramanian:** Correction of the manuscript; **P. Pretheep Kumar:** Reference part.

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Optimization of enzymatic clarification of sapota (*Achras sapota* L.) juice using response surface methodology

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Abstract

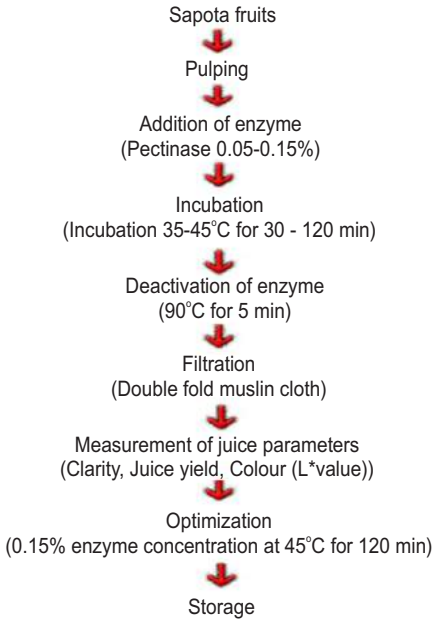
Aim: The present study aimed to develop enzymatic clarified sapota (*Achras sapota* L.) juice beverage under optimized conditions for future scale up.

Methodology: In this study, the ripened sapota fruits PKM 1 variety were ground to pulp and pulp was mixed with 0.05-0.15% pectinase enzyme and incubated at 35°C-45°C for 30-120 min. After incubation period the enzyme was deactivated by placing the pulp in warm water bath at 90°C for 3 min. Optimal enzymatic clarified juice was produced by pectinase enzyme by response surface methodology.

Results: The absorbance values decreased with increasing incubation time at fixed temperature. Incubation time showed a significant and $p<0.05$ negative effect on L* value at linear terms. At fixed temperature, the L* value increased with increasing enzyme concentration. Significant regression models proved that the changes in clarity, juice yield and colour (L*values) when compared to the independent variables demonstrated, coefficient of determination, R^2 greater than 0.8.

Interpretation: Optimization conditions like minimum clarity, maximum juice yield and maximum color (L* value) can be applied in the production of sapota juice for commercial use.

Key words: *Achras sapota*, Clarified juice, Pectinase enzyme, Response surface methodology



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Introduction

India is one of the major producer of sapota fruits, followed by Mexico, Guatemala and Venezuela. India is the second largest producer in the production of Fruits and vegetable and its annual production is around 80,000 - 85,000 MT from the area of about 6982 ha and productivity of 11.64 MT per hectare. Nutrient contents of sapota fruits consist (21.49 g) carbohydrates, (0.7 g) protein, (1.1 g) fat, (73.7 g) moisture, (27 mg) phosphorus, (28 mg) calcium, (2 mg) Iron and (6 mg) ascorbic acid (Gopalan *et al.,* 2005). Value added products like jams, jellies, osmodehydrated slices, sweet chutney, squash, sapota milk shake, dried sapota pieces, nectar, pickle, blended sapota drinks, candy and fermented wines are prepared from sapota. (Gautam and Chundawat, 1998). Sapota reduces inflammation and pain prevents gastritis, reflux oesophagitis and bowel disorders. Sapota strengthens the intestines, boosts immunity and prevents from bacterial infections as it is rich in Vitamin C.

Pregnant women are advised to eaten sapota for high nutritional content as it reduces weakness, nausea and dizziness and prevents anaemia. It is also useful in constipation and piles (Baskar *et al.,* 2020). As beverage industry is growing every year, it is necessary to develop technology for fruit juice based beverages with assured quality. Response surface methodology is a statistical method mostly used for optimizing products in the food industry (Frank, 2001; Luciane *et al.,* 2001; Pietrasik and Li-chan, 2002). It involves designing experiments, selecting variables in experimental design, fitting mathematical models and finally selecting variables by optimizing the response (Nithya *et al.,* 2016). Using Design Expert Software, RSM can be performed for optimization of product ingredients.

Along with optimization, RSM can also be used to find the effect of correlation between the inputs on the response, wherein more than one response can be studied (Josephine *et al.,* 2014). Response surface methodology, minimizes the number of experimental trials required to evaluate multiple parameters and their interactions. Recently, the clarified fruit juice products are in gread demand in the market. About 25 to 30 per cent of fruits and vegetables are lost annually post harvest due to spoilage, inadequate handling, transport, storage, lack of processing facilities and non-availability of market (Bhat, 2000).

During season, the selling price of sapota is less than Rs. 10 kg^{-1}. Sapota juices are used as nutritional and nutraceutical health beverage, as they are rich in polyphenols. Enzymatically clarified sapota juice beverage can be used as a health promoting beverage because of its multifunctional properties (Kulkarni *et al.,* 2007). In view of the above, this study was carried out to produce enzymatically clarified sapota juice by response surface methodology. Hence, an experiment was undertaken under optimized conditions (incubation time, temperature and enzyme concentration) for preparing of enzymatic clarified sapota juice using RSM.

Materials and Methods

The study was conducted in the Department of Food Science and Nutrition, Community Science College and Research Institute, Tamil Nadu Agricultural University, Madurai, India, during the year 2017 to 2020. Good quality and medium size fruits of sapota (*Achras sapota* L.) variety PKM 1 fruits were harvested, cleaned and allowed to ripen for one week at ambient temperature. The fruits were procured from the Horticultural College and Research Institute, Periyakulam, TNAU, Tamil Nadu, India. The ripened sapota fruits were washed, dried, peeled, deseeded and pulped using a food processor for two minutes. Commercial enzyme Pectinex Ultra SPL (Novozyme, Denmark) was used as clarifying agent for preparing clarified sapota juice. Fresh sapota pulp was mixed with 0.05-0.15 per cent pectinase enzyme and incubated at 35°C-45°C for 30-120 minutes (Vijayanad, *et al.,* 2010; Relekar *et al.,* 2011). After incubation period, sapota fruit pulp enzyme content was deactivated through water bath at the temperature of 90°C for 5 min. From the sapota pulp, juice was filtered through double fold muslin cloth and the juice was extracted and used for further treatments.

Clarity: The pectin molecules was breakdown by pectolytic enzymes, which make easy the formation of pectin–protein flocs, leaving a pure supernatant and extensively removing the colloidal phase of the juices. In the clarified juice, clarity is an important index. Clarity value was measured on a Elico UV–VIS spectrophotometer (SL 210 UV VIS spectrophotometer, Japan) and the absorbance was read at 660 nm. In a sample distilled water were used as reference.

Juice yield: Clarified sapota juice yield was estimated as a percentage of juice obtained from a unit weight of the sample. Sapota juice yield was calculated by the following formulae:

$$\text{Juice yield \%} = \frac{\text{Weight of clear juice}}{\text{Weight of sample}} * 100$$

Colour (L* value): Hunter Laboratory Calorimeter (model SN 7877, Ultrascan, Hunter Associates Laboratory, Inc., Virginia) was used to measure the colour (L*) value of the clarified juice (Sin *et al.,* 2006). where +LÕ is the lightness and L is the darkness (Busch-Kschiewan *et al.,* 2004). To measure the colour value of the samples, the instrument was first calibrated by keeping black and white standard pieces. The standard value was measured and followed by the sample colour value was measured by filling the fruit juice in the transparent pieces and values were noted. Change in the colour values from the samples to the standard were observed and recorded.

The mean of three trials was reported. Observed colour (*L** values) showed lightness / darkness and ranged from 0 (black) to 100 (white) with upper values corresponding to more lightness. Object's colour dimensions namely as *a** and *b** values, *a** indicated red (+a) to greenness (-a) and *b** indicated yellow

(+b) to blueness (-b). Higher *a** values indicated more redness while higher *b** values indicated more yellowness.

Experimental design: In this experiment, optimized conditions for the enzymatic clarification of sapota juice was determined by Response Surface Methodology (RSM). It highlights the modeling and analysis of the problem where response of interest is subjective by several variables and the objective is to optimize this response by Montgomery (2008). For designing this experiment, a central composite design was employed. D - Optimal Design was used to study the interaction of process variables by applying RSM (Bezerra, 2008). Response surface methodology is a number of experimental trials needed to provide sufficient information for statistically suitable results. A five-level and three-factor central composite rotatable design was used by Myers & Montgomery (2002). In this experiment, the lowest and highest values for effect of enzyme concentration 0.05% to 0.15% , incubation time were set at 30 to 120 min and temperature at 35°C to 45°C.

Statistical analyses were carried out by using ECHIP Software Version 6. (Echip Inc., Hockessin, Delaware, USA) (Echip Software, 1993). A central composite rotational design was used in the experiment by Cochran and cox (1957) to study the combined effect of three independent variables, *i.e.,* incubation time, temperature and enzyme concentration. X1, X2, and X3 are the three independent variables. An_1, 0, and +1 three levels were called as independent variables. These three independent and dependent variables were used for the treatment of enzyme clarified juice (Baumann, 1981). Nineteen combinations and five replicates were used in random order and followed to a CCD design for these selected variables. Clarity (y_1), juice yield (y_2) and color (L*) value (y3) were used to measure the dependent variables (y) for sapota juice. Three dependent variables and Independent variables were expressed as response function separately, as follows.

$$Y_0 = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3 + b_{11}x_1^2 + b_{22}x_2^2 + b_{33}x_3^2$$

In this experiment, data were expressed as b0 (constant term), b_1, b_2 and b_3 (linear coefficient), b_{11}, b_{22} and b_{33} (quadratic coefficient), and b_1^2, b_1^3 and b_2^3 (interactive coefficient). The polynomial functions and its importance were expressed as statistically using F-value at a probability level of 0.001, 0.01 or 0.05. Contour maps produced from the regression models were graphically expressed by regression coefficients. One variable constant at the center point and other variables within the experimental range were used to produce graphical three dimensional (3D) plots.

Results and Discussion

R^2, is the ratio of the variation explained by the given independent variables to the total variation. Also it establishes the degree of fit i.e., coefficient of determination, (Haber and Runyon, 1977). Coefficient of determination is also the measure of the variability in the response variables and accounts for the regression analysis (Mclaren *et al.,* 1977). Unity and better empirical model fits the actual data in R^2. Behaviour variation explaining minor value of R^2, less consequence and dependent variables in the model were used. Table 2 showes that the response surface model developed for all the response variables were satisfactory from the analysis of variance in these three variables. The R^2 values were more than 0.8, its stated that the regression models in the response variables were well explained in this study.

The concentration of enzyme as its linear effect was negative and its quadratic effect was positive for clarity. Interaction effect between incubation time and enzyme concentration at $p<0.05$ was positive and significant, The enzyme concentration and incubation time were main reason for clarity. At fixed temperature, enzyme concentration and time showed clarity (Fig.1). Absorbance values were decreased, indicating that there is an increase in enzyme concentration. Less absorbance value was observed at more enzyme concentration. The clarified sapota juice recorded Lower absorbance values. Increase in enzyme concentration resulted in increased rate of clarification, reduced electrostatic magnetism between the particles. These particles aggregated to larger particles which eventually settled down exposing the part of the positively charged protein beneath. Increasing incubation time at fixed temperature decreased the absorbance values.

The linear negative effect (Table 1) of incubation time at $p<0.01$ level was leading. Incubation time, temperature and enzyme concentration are important factors for obtaining to clear clarified juice (Kilara, 1982). The model F value was highly significant ($p<0.0001$) as showed by the analysis of variance. The higher F value could occur due to noise at 0.01%. Probability > of F value lesser than 0.05 indicated that estimated model are significant. Higher clarified juice yield was obtained from the following conditions (coded form): Enzyme concentration was used to 0.15g/100 g, 120 minutes incubation time at 45°C incubation temperature. Results also stated same that the enzymatically hydrolyzed clarified guava juice yield was increased and it can be due to the action of the pectinases in the guava pulp. It also Increased the clarified juice yield due to the action of pectinases humiliate pectic substances (Pilnik&Voragen, 1993; Diwan&Shukla, 2005). The first judgement of a clarified juice quality is colour. clarified juice colour (L* value) measures the lightness of the product and the value ranges should be as high as possible. The clarified juice product was dark coloured meaning that it is deterioriating the product and less appealing by the consumers. In this experiment, incubation time showed a significant level $p < 0.05$ and a negative effect on colour (L* value) at linear terms. The clarified juice colour (L*) value at fixed temperature with enzyme concentration and incubation time are presented in Fig. 3. The colour (L* value) of the clarified juice were gradually increased with increase in enzyme concentration at constant temperature.

Table. 1: Effect of incubation time, temperature and enzyme concentration on three dependent variables for enzymatic clarified sapota juice

	Independent variables		Dependent variables		
Time (Minutes)	**Temperature (°C)**	**Enzyme conc. (%)**	**Clarity (abs)**	**Juice Yield (%)**	**Color (L*value)**
X_1	X_2	X_3	b_1	b_2	b_3
75.00	40.00	0.10	0.2236	55	35.94
30.00	45.00	0.05	0.3228	51	33.54
120.00	45.00	0.05	0.1795	56	36.87
30.00	35.00	0.05	0.3114	49	32.65
30.00	35.00	0.15	0.2931	53	31.33
75.00	31.59	0.10	0.2509	54	34.05
120.00	35.00	0.05	0.1795	56	36.58
75.00	40.00	0.10	0.2245	56	35.94
75.00	48.41	0.10	0.2416	58	34.98
150.68	40.00	0.10	0.1934	58	37.62
120.00	45.00	0.15	0.1495	61	39.28
75.00	40.00	0.10	0.2131	55	35.63
75.00	40.00	0.10	0.2235	55	35.94
75.00	40.00	0.18	0.2445	59	34.96
75.00	40.00	0.02	0.3217	49	33.56
120.00	35.00	0.15	0.1502	59	38.77
30.00	45.00	0.15	0.3012	57	35.45
75.00	40.00	0.10	0.2132	55	35.63
75.00	40.00	0.10	0.2131	56	35.94

Table. 2: Regression coefficient, R2 and Probability values for three responses for clarification of sapota juice

Regression coefficient	Clarity (abs)	Juice yield (%)	Colour (L*value)
b_0	0.44527	74.47332	17.10309
b_1	-2.47404	0.28740	0.16111
b_2	1.29075	-1.94811	0.53926
b_3	-1.24083	-17.36977	-56.73834
b_1^2	1.00300	-7.18792	-2.46809
b_2^2	1.19857	0.026631	-5.35441
b_3^2	5.33123	-16.53609	-89.60659
b_{12}	-1.2222	-2.2222	-2.33889
b_{13}	-1.07778	-0.1111	0.22278
b_{23}	-2.0000	2.0000	1.72500
R^2	0.9133	0.9159	0.9035
P or probability	0.0000	0.0000	0.0000

*Significant at 0.001 level; **Significant at 0.01 level and ***Significant at 0.05 level

The clarified juice colour (L* value) decreased initially up to 80 min and later gradually increased due to formation of protein–tannin complex as incubation time increased. In this experiment, the most important factor was enzyme concentration and influenced the clarification of sapota fruit juice and its enzymatic treatment. Temperature was the least significant variable and it did not show significant effects on linear, quadratic and interactive regressions model in the clarified juice. In this study, moderate temperature were used for the preparation of enzymatic clarification of sapota juice. In this experiment, optimum clarification condition of juice was explained by superimposing contour plots for three response variables. It was found that there is the good resulted treatment combination from the variables and its related to the response variable functions (clarity, juice yield and color) and cost of the enzyme were to be found. In this experiment, enzyme treatment was found most suitable for the optimum condition to prepare clarification of sapota juice through superimposing the contour plots of all three response variables (Sin *et al.*, 2006; Vandana *et al.*, 2006;Vijayanad, *et al.*, 2010; Norjana *et al.*, 2011). It is revealed that the final condition were to be considered optimum if the absorbance value were as low as possible while the clarity value,

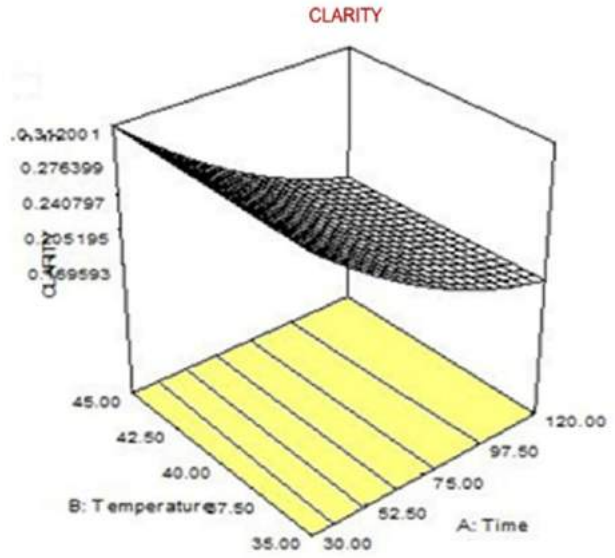

Fig. 1: Response surface for clarity of sapota juice as a function of temperature and enzyme concentration for 120 min at 45°C.

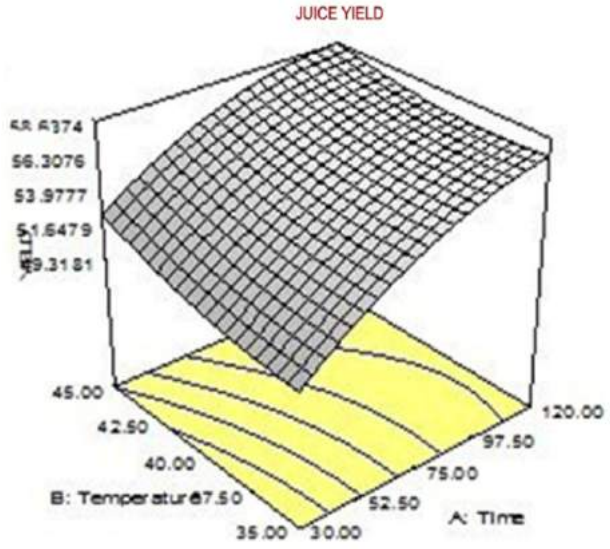

Fig. 2: Response surface for yield of sapota juice as a function of temperature and enzyme concentration for 120 min at 45°C.

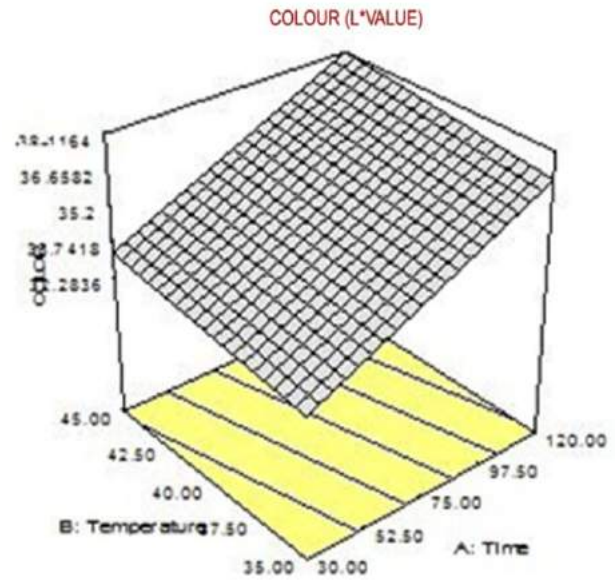

Fig. 3: Response surface for color L*value of sapota juice as a function of temperature and enzyme concentration for 120 min at 45°C.

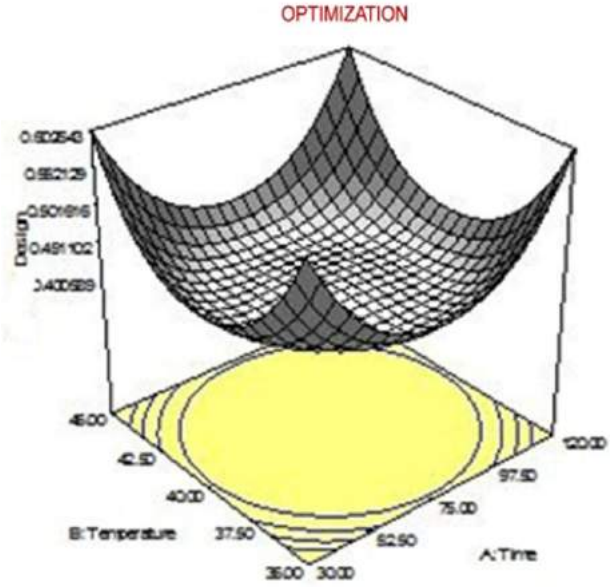

Fig. 4: Response surface for optimization of sapota juice as a function of temperature and enzyme concentration for 120 min at 45°C.

colour (L* value) absorbance value were slightly high and juice yield was as high as possible. In this experiment, graphical optimization parameter were found as follows: (a) minimum clarity (b) maximum juice yield and (c) maximum color (L* value). The response function like clarity, juice yield and color (L* value (Figs. 1–3)) were computer generated plot and using the parameter for an optimum condition of the superimposed plot as shown in Fig. 4. The response function variables were selected as the important index of the physical parameters (clarity, juice yield and color (L* value) of the clarified juice. (Figs. 1–3), graphical representation of the optimum conditions for the response function variables, while the optimum combined condition for the

clarification of sapota juice was found to be at 0.15% enzyme concentration at 45°C for 120 min as shown in Fig. 4.

The increasing demand for soft drinks offers a great scope for development of natural nutrient rich beverages. These drinks help the consumers in having an option which has negligible synthetic chemicals and also has immense health benefits. The growing consumer awareness particularly with regard to health benefits of the products consumed has led to an exponential growth in the demand for food and also beverages made from natural sources that contain ingredients preferably having nutraceutical properties. As a ready to serve beverage with distinct sapota flavor and taste, the technology can be widely adopted from the food and beverage industry where the enzyme clarified juice has ready marketability.

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Add-on Information

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Bio-safety assessment of nanozeolites of varying size and doses on soil beneficial microorganisms

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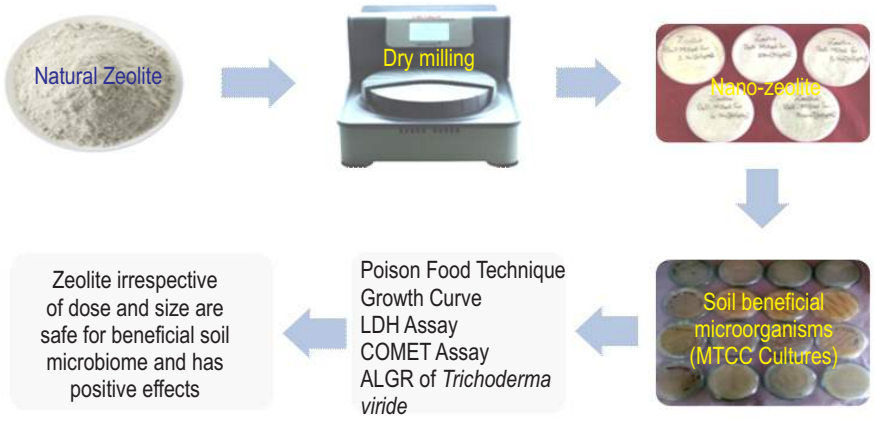
Abstract

Aim: To evaluate the toxic effects of nano-zeolites on soil beneficial microorganisms.

Methodology: This study involved dry milling of zeolites at varying parameters to obtain dry-milled zeolites of four size regimes such as 10-100 nm, 200-400 nm, 500-1000 nm and 1000-2000 nm that were tested at incremental concentrations such as 100 ppm, 1000 ppm and 2000 ppm along with control on various soil beneficial microorganisms. Three replications were done for each treatment with factorial completely randomized design. The poison food technique, growth curve assessment followed by cytotoxicity and genotoxicity studies on all four bacterial genera treated with zeolites of varying sizes and doses were undertaken. The effects of zeolite on average linear growth rate (ALGR) of biocontrol agent, *Trichoderma viride* were also studied.



Results: The effect of zeolites tested on four bacterial genera viz. *Azotobacter chroococcum, Rhizobium leguminosarum, Bacillus megaterium* and *Pseudomonas fluorescens* using poison food technique and growth curve revealed that zeolites regardless of size or concentration had positively influenced the growth dynamics of all four bacteria tested. The effect of zeolite on average linear growth rate (ALGR) of *Trichoderma viride* also indicated that incremental dose of zeolite had a positive effect. Lactose dehydrogenase revealed that 2000 ppm nano-zeolite exhibited cytotoxic effects on soil beneficial micro-organisms tested. On the other hand, comet assay demonstrated no quantifiable DNA damage in nano-zeolite treated cells in comparison to control cultures.

Interpretation: This study unequivocally demonstrated that zeolites of size greater than 200-400 nm, irrespective of doses even up to 2000 ppm are quite safe for soil beneficial microbes.

Key words: Bacteria, Comet assay, Cytotoxicity, Nano-zeolite, *Trichoderma viride*

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Introduction

Interventions in the size regime of 'nano' are being exploited in varied sectors for resolving several issues that cannot be achieved either through conventional methods or by bulk materials (Subramanian and Tarafdar, 2011). High surface area to mass ratio is a unique property that a material acquires when converted into nanomaterials. This characteristic feature indicate changes in other properties such as electrical, optical, mechanical etc., that makes the nanomaterials best candidates for novel applications (Vuong and Do, 2015; Kolhatkar *et al.,* 2013). Naturally occurring minerals like zeolites are promising material that are employed in a wide range of application. Zeolites are naturally occurring crystalline aluminosilicates with unique properties such as large surface area, defined pores and structures, high thermal and mechanical resistance with tunable chemical composition and most importantly, they are recoverable and reusable (Chen *et al.,* 2012; Mintova *et al.,* 2013). The uniqueness of zeolite comes from its ordered three-dimensional structures with microporous channels of interconnected tetrahedral, comprised of Al, Si and oxygen atoms.

The general formula of zeolite is $Me_{2/n}$ O. Al_2O_3.xS_iO_2. $_yH_2O$ (Bogdanov *et al.,* 2009). Agricultural scientists find nano-zeolites as a promising material for the development of better soil conditioners, growth medium in nurseries and nano fertilizers (Subramanian and Sharmila Rahale, 2012; Subramanian *et al.,* 2015; Subramanian and Thirunavukkarasu, 2017; Suratman *et al.,* 2020). Nano zeolites are widely researched in several fields including medicine (Derakhshankhah *et al*., 2020) and hence, large quantities are likely to be produced in near future. Though nanoparticles in general exhibit novel properties as a result of increased surface area and reactivity, it is this transformation that is also a cause for concern owing to increasing reports of toxicity pronounced by several nanomaterials (Rajkishore *et al.,* 2013). Thus, this study was focused on assessing the effect of size reduced zeolites at varying concentrations on growth, cytotoxicity and genotoxicity of four beneficial bacteria and average linear growth curve of *Trichoderma viride*.

Materials and Methods

Experimental site and source materials: This investigation was undertaken at the Department of Nano Science & Technology, TNAU, Coimbatore to assess the biosafety of nano-zeolites on soil beneficial microorganisms. Zeolites used for the study was fine textured naturally occurring (Clinoptilolite) that was obtained from GM Chemicals, Ahmedabad and the beneficial microorganisms (*Trichoderma viride, Bacillus megaterium, Pseudomonas fluorescens, Rhizobium leguminosarum*) were collected from Microbial Type Culture Collection and Gene bank (MTCC), Chandigarh and *Azotobacter chroococcum* was obtained from the Department of Microbiology, TNAU, Coimbatore.

Synthesis and characterization of nano-zeolite by ball milling: Nano-zeolites were synthesized using ball milling, a top-down approach (Mechanical synthesis). Process was optimized and our developed standard operational protocol was adopted (Sivashankari *et al.,* 2019). Ball milling was performed by utilizing the planetary ball milling (FRITSCH, Germany, Model: Pulverisette 7) available at the Department of Nano Science and Technology, TNAU, Coimbatore. Ball milling at 400 rpm for 5 hrs produced nano-zeolite under the size regime of 10-100 nm. Particle size was observed using Nano Particle Size Analyzer (Model: HORIBA-SZ-100), Scanning Electron Microscope (FEI, Quenta 250) and Transmission Electron Microscope (FEI Technai Sprit).

The particle size distribution of zeolite milled at 2 hr, 3 hr, 4 hr and 5 hr were in the range of 300-550, 200-400, 100-200 and 10-100 nm, respectively. The zeta potential was measured between -30mV to -60mV and all the milled samples including natural zeolites (H0) were found stable. SEM and TEM imaging of samples revealed that natural zeolites were cubic shaped and nano-zeolites as spherical. EDAX data showed that the zeolites have Si/Al ratio of 4.14 indicating that the study material is clinoptilolite type of zeolite. FTIR peaks clearly indicates a slight frequency shift in nano-zeolites. In XRD spectra of nano-zeolite, eight prominent peaks that were recorded in natural zeolites were not observed, indicating that there was an intense reduction in crystalline nature of zeolite after ball milling process. SEM image showing zeolites milled at different hours are presented in Fig.1.

Biosafety studies: Pour plate method was adopted to assess the effects of varied size and concentration of zeolites on microbes. A fixed amount of inoculum from 10^{-5} dilution was taken and placed in the center of sterile petriplate to which approximately, 15 ml of warm/partially cooled melted nutrient agar containing zeolites at different concentrations (100ppm, 1000ppm and 2000ppm) and varied size range (10-100nm, 200-400nm, 500-1000 nm, and natural zeolite of around 1-2µm) was added and mixed well with the inoculum. Once the medium was solidified, the plates were kept inverted in an incubator at 30°C for 18-24 hr. Then each colony was carefully counted using magnifying lens (at 18 and 24 hr) and each of colony represents a colony forming unit (CFU). The number of colonies counted in each treated test samples were further determined by the formula: CFU/ml= No. of colonies × dilution factor

The effect of zeolites on the growth dynamics of microbial population was determined by turbidometric technique by analyzing the growth trend of bacterial cell in a liquid media. Spectrophotometer was employed to observe changes in the optical density (OD) over the period of time for plotting a growth curve. A 60 ml sterilized broth was taken in 100 ml conical flasks to which zeolites (respective sizes) were added. Then it was mixed well to achieve homogenous dispersion. Subsequently, 1ml of 10^{-5} diluted culture grown in liquid medium was inoculated in each of the conical flask. Immediately, the inoculated samples (4 ml of it transferred to a clean cuvette) were analyzed for OD value at zero hour. The sterile broth alone was used as blank. The same procedure was repeated for all the bacterial cultures at a time interval of 3 hrs upto 32 hrs. All the readings were recorded at

660nm. The readings obtained were plotted in the graph with time on X-axis and OD value as Y-axis. Lactate Dehydrogenase Assay (Decker and Lohmann, 1988) was performed to assess the cytotoxicity effects of experimental treatments (Nano and Natural Zeolites) on four beneficial microorganisms *viz., Pseudomonas, Bacillus*, *Azotobacter* and *Rhizobium*. Cultured cells were incubated in individual wells of ELISA plate that were exposed to different forms of zeolites to assess cytotoxicity potential.

The released LDH enzyme was subsequently quantified using ThermoFisher Pierce LDH Cytotoxicity Assay Kit (Cat#88953), USA by following the manufacture's protocol. ELISA plates containing 50 ml of bacterial cultures incubated with zeolites (nano to microscale) and 50 ml of reaction buffer were kept undisturbed in dark condition for 30 min. Then, 50 ml of stop buffer was added to each wells of ELISA plates and absorbance value was recorded at 490 nm and 680 nm immediately after adding of stop buffers using ELISA plate reader (SpectraMax i3X) and data acquisition using SoftMax Pro ver.6.5.1 of Molecular Devices LLC, USA. The maximum LDH activity of controls and Spontaneous LDH activity controls for each microorganism were calculated based on the mean of three replicates. Per cent cytotoxic was determined by the formula:

$$\text{Percent Cytotoxicity} = \frac{\text{Nanotreated LDH activity - Spontaneous LDH activity}}{\text{Maximum LDH activity - Spontaenous LDH activity}} \times 100$$

COMET Assay was performed to assess the genotoxic effects of nano-zeolite on the *Rhizobium* cultures by the action of mobility of nano particles into the cell / nuclear membrane resulting in irreversible DNA damage (Tice *et al.,* 2000).The magnitude of DNA damage was quantified using Gel Doc™ XR+, BioRad, USA and observation on DNA damage was recorded. The Average Linear Growth Rate (ALGR) of *Trichoderma viride* treated with zeolites was also measured. For all the treatments, 20 ml of medium containing measured amount of zeolites (of varied size and dose) was poured in each petriplate (36 + 3 plates for treatment and control). As soon as the medium was solidified, a 5 mm mycelium block was placed upside down at the center of each petriplate. The petriplates were then incubated in growth chamber maintained at room temperature (27±2°C).In order to measure ALGR of *Trichoderma*, the linear growth (mm) of the fungi was measured by obtaining the average of three diameters taken from each plate. The standard formula used to measure the Average Linear Growth Rate is:

"ALGR (mm/day) = (Colony diameter after 3 days of inoculum - Initial colony diameter of inoculum)/3"

Statistical Analysis: The data obtained from these experiments were statistically analyzed using AGRES software.

Table 1: Treatments conducted to study the toxic effects of zeolites of varying sizes at varying doses

T0	Control	Without zeolite
T1S1	100 ppm	10-100 nm
T1S2		200-400 nm
T1S3		500-1000 nm
T1S4		1000-2000 nm (Natural zeolite)
T2S1	1000 ppm	10-100 nm
T2S2		200-400 nm
T2S3		500-1000 nm
T2S4		1000-2000 nm (Natural zeolite)
T3S1	2000 ppm	10-100 nm
T3S2		200-400 nm
T3S3		500-1000 nm
T3S4		1000-2000 nm (Natural zeolite)

Results and Discussion

Poison food technique was employed to study the effect of varied sizes and concentration of zeolites on population count of beneficial microorganisms (Table. 2 and Fig. 2). In general, the population counts were significantly higher in zeolite and nano-zeolites enriched medium compared to control. The plate count was significantly highest in the treatment T_3S_4 for two bacterial genera such as *Rhizobium* and *Pseudomonas*, while the highest counts were recorded under T3S3 (500-1000 nm @ 2000 ppm) for other two bacterial genera *Azotobacter* and *Bacillus* when compared with control plates. *A. chroococcum*, under the influence of T_3S_3 and T_3S_1 sized zeolites, recorded 39% and 34% more colonies compared with control plates in which no zeolites were added in the growth medium. For *B. megaterium*, the increase in plate count was 41% under T3S3 and 37% under T3S1, compared to control. Overall, both natural and nano-zeolites increased the microbial growth and among the four micro-organisms, the highest growth enhancement was recorded in *R. leguminosarum*, followed by *P. fluorescens*, *A. Chroococcum* and *B. megaterium*.

The effect of zeolite on growth dynamics of four different beneficial microorganisms are shown Fig. 3. The growth curve of all four bacterial genera followed the usual trend of lag phase, log or exponential phase, stationary phase and death phase and the trend was similar for all the treatments. The growth trend of all four bacteria showed that the zeolite irrespective of its size range had no toxic effects on bacterial cells. Instead, the growth in treated ones were found to be significantly higher than control. The maximum absorbance was observed in *P. fluorescens* after 32 hrs (0.788), followed by *A. chroococcum* (0.591), *R. leguminosarum* (0.587) and *B. megaterium* (0.587) in treatment T_3S_4. Such increase in microbial population counts and biomass in the zeolite treated medium suggests that substrate use efficiency was enhanced as result of zeolites. This is attributed to the fact that zeolites increase carbon incorporation into microbial biomass and our observations are in agreement with Chander and Joergensen (2002) and Heinrichs *et al.* (1986) who reported that zeolites promote glucose decomposition thereby increases microbial biomass. In addition, zeolite as a mineral might have triggered cell division and contributed for increased population counts. Similar responses as a result of clay mineral such as kaolinite have been reported by Courvoisier and Dukan (2009) who observed rapid cell division and increased microbial population after adding these materials. It is also speculated that

Table 2: Effect of zeolite on population count of beneficial microorganisms (24th hour) (Xx10^{x} cfus ml^{-1})

Treatment		*Azotobacter chroococcum*	*Bacillus megaterium*	*Pseudomonas fluorescens*	*Rhizobium leguminosarum*
Dose	Size				
T0	Control	321.15	308.15	324.00	338.00
T1	S1	334.26	294.33	367.68	315.63
	S2	356.00	312.00	392.70	367.60
	S3	347.66	302.44	365.00	374.80
	S4	362.33	323.15	343.33	377.34
T2	S1	359.00	383.00	332.33	443.00
	S2	363.00	378.22	367.33	452.66
	S3	347.66	416.66	374.00	438.00
	S4	362.33	402.09	399.56	465.73
T3	S1	437.66	425.12	433.00	454.22
	S2	424.00	419.22	426.00	458.66
	S3	453.00	448.00	448.66	451.00
	S4	449.00	437.00	458.00	472.00
SEd	0.224	0.206	0.249	0.242	
CD (0.05%)	0.463	0.425	0.515	0.499	

Table 3: Effect of zeolite on linear growth rate of *Trichoderma viride*

Treatment		Average linear growth rate (mm per day)	
Dose	Size		
T0	Control	21.50	
T1	S1	21.85	
	S2	22.91	
	S3	22.88	
	S4	21.03	
T2	S1	20.87	
	S2	23.32	
	S3	22.64	
	S4	21.91	
T3	S1	22.50	
	S2	22.17	
	S3	23.71	
	S4	23.50	
		SEd	CD (5%)
Dose		0.2645	0.5819
Size		0.3054	0.6718
Dose × Size		0.5290	1.1638
Ctrl vs Trtd		0.3893	0.8564

leaching of metal nutrients from the zeolite framework might have also provided additional nutrients in the medium favouring microbial growth. Furthermore, impurities as nutrients over the surface of zeolites could have contributed for microbial metabolism since zeolites used in this study for milling process were also natural zeolites. These statements are in accordance with the reports of Hrenovic *et al.* (2003) who reported that metals leached from zeolites may play a role as nutrients. Such discussions may also dispel the fact that zeolites are inert carriers of nutrients, since studies on microbe-mineral interactions highlight that micro-organisms develop several strategies to attack minerals for extracting nutrients from them for metabolism (Cuadros, 2017). On the other hand, zeolite might have facilitated for adherence/immobilization of microbial cells on its framework thereby providing a conducive platform for nutrient uptake and metabolism. This reason is in conformity with the reports of Vieira and Melo (1995) who observed that positively charged ion exchange sites over zeolite enhanced the bacterial growth. Though zeolites in general were found to enhance the growth in micro-organisms, the increase in population count and biomass by nano-zeolites was relatively lower compared to zeolites of the size range 1000-2000 nm. This is attributable to the fact that ball milling process had changed the structure of nano-zeolites which could be the reason for reduction in the bacterial colonies compared to natural zeolites. In addition, the mechanical synthesis of nano-zeolites might have disturbed the framework of zeolites leading to release of alumina and this component could have decreased the positive effects. Similar observations have been reported by Kihara *et al.* (2011). Overall, the positive effects of natural zeolites might have been offset by the size reduction to nano range.

The cytotoxicity effects of treatments amended with natural zeolite and nano-zeolite were quantified using Lactose Dehydrogenase Assay and the results obtained are presented in Fig.4. In comparison of all the treatments exposed to zeolites of varying sizes and concentration, treatments T_3S_1 showed the presence of toxicity with varying degree among the four beneficial micro organisms. The maximum cytotoxicity (%) was recorded in *P. fluorescens* (51.0%), followed by *R. leguminisarum* (46.4%), *B. megaterium* (44.5%) and *A. chroococcum* (25.8%). Among four bacterial species that were exposed to zeolite, *Pseudomonas* sp. was more prone to cytotoxicity irrespective of treatments (varying size and concentration) which is evident from the highest level of LDH content in the culture broth of all treatments. Further, *Azotobacter* sp. demonstrated comparatively higher level of resistance to cytotoxicity regardless of size or concentration. It

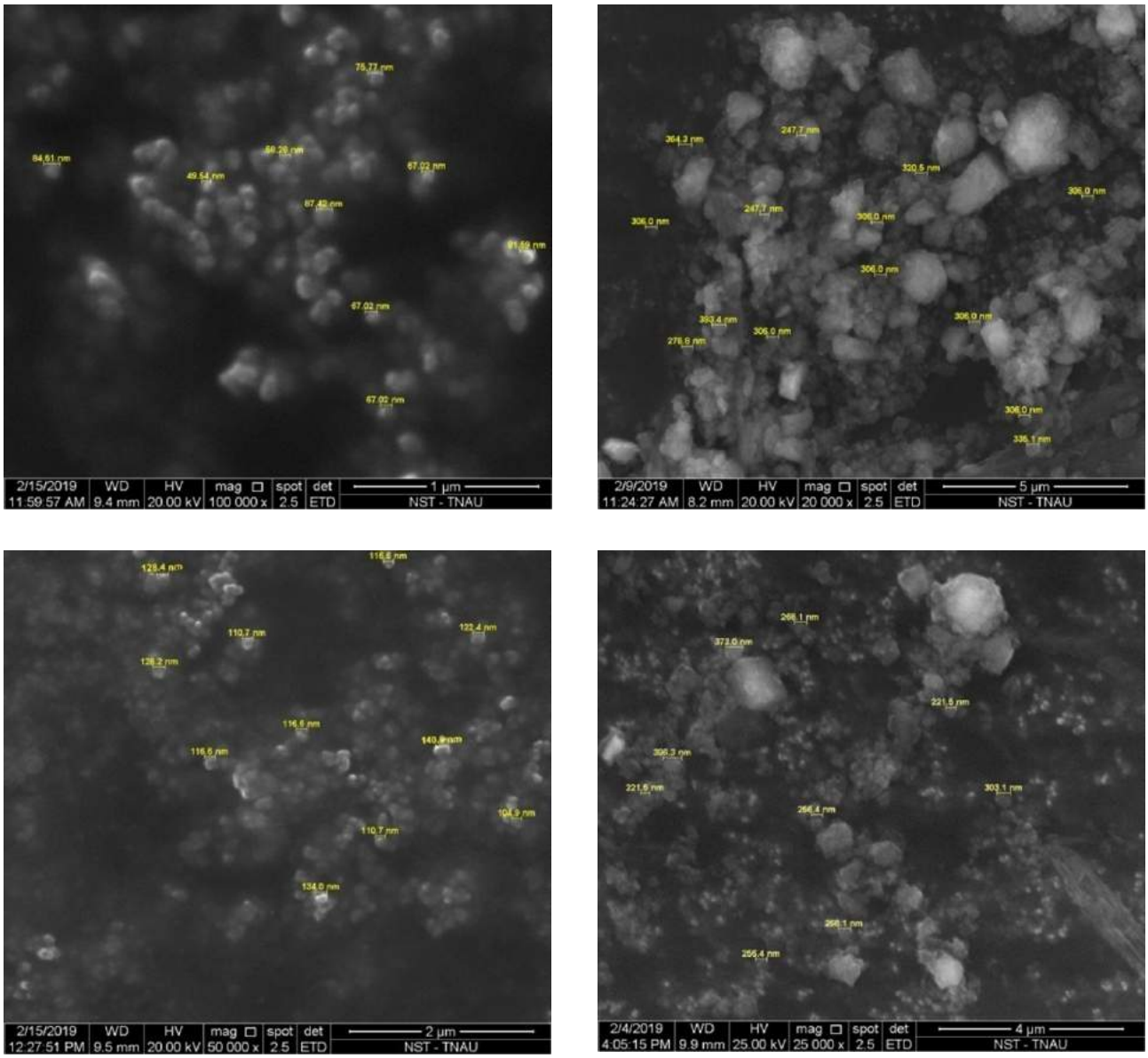

Fig.1: SEM image showing zeolite milled at different time duration: (a) milled for 2 hr (b) milled for 3 hr (c) milled for 4 hr and (d) milled for 5 hr.

was quite interesting to observe that cytotoxicity assay could provide better insights on the size and concentration dependent toxicity of zeolites. Poison plate technique revealed that zeolites regardless of size or concentration significantly enhanced the microbial population. In contrary, the data on LDH assay suggested that 2000 ppm nano-zeolite exhibited cytotoxic effects on soil beneficial micro-organisms. This is evident from the increased surface area and change in crystal shapes of nano-zeolites might have contributed for cytotoxic effects. Our observations are in accordance with the studies of Kihara *et al.* (2011) and Thomassen *et al.* (2012) who have reported *in-vitro* cytotoxicity caused by nano-zeolites. It is also due to increased concentration (2000ppm) of any material could possibly increase the cell lysis process (Amanchi and Hussain, 2010). Furthermore, it is understandable that microbes in nano-zeolite treated petriplates might have overcome the toxic effects since nutrient medium could have supported better cell proliferation. Nevertheless, the bacterial cells incubated for LDH assay had higher interactions with nano-zeolites, especially at the highest dose (2000 ppm) and it could capture the cytotoxicity suggesting that this assay is more reliable to quantify even the minimal events of cell lysis.

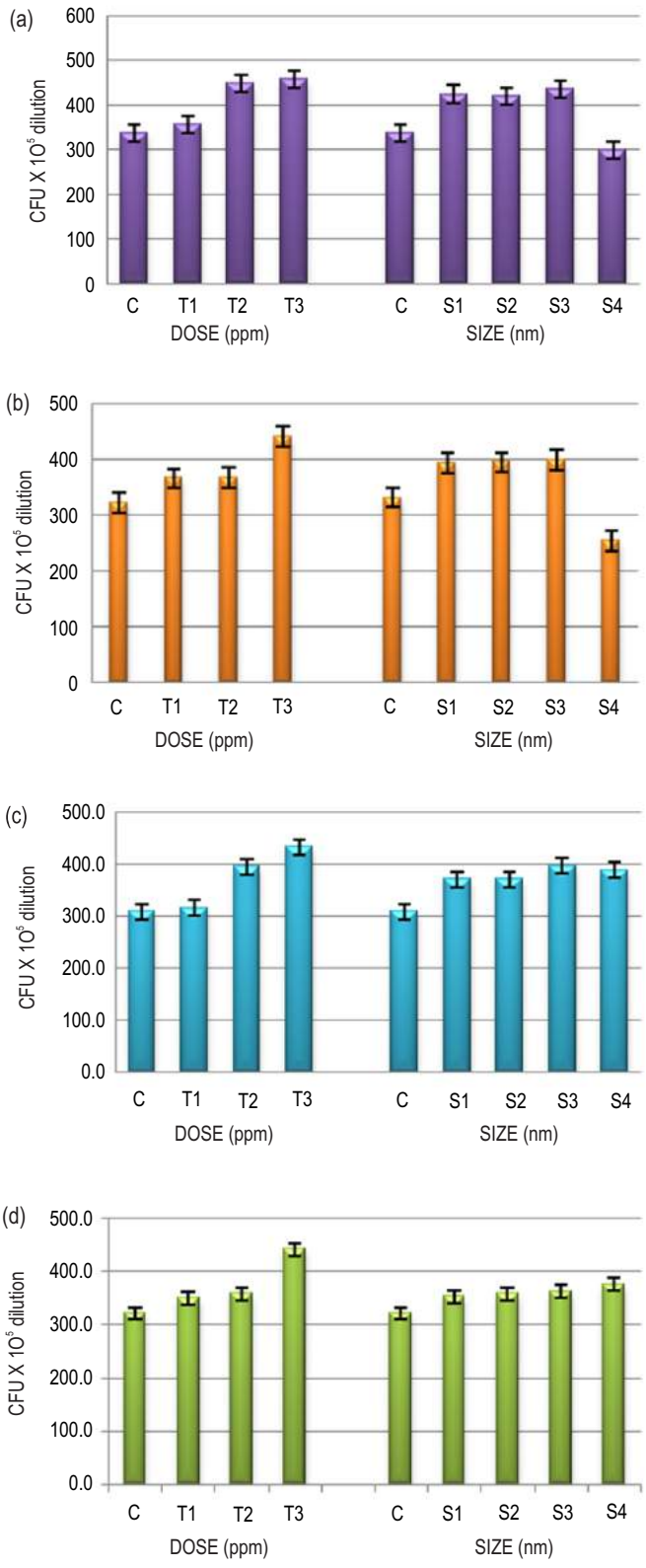

Fig. 2: Total plate counts (CFU) in zeolite treated test organisms: (a) *Azotobacter chroococcum;* (b) *Bacillus megaterium;* (c) *Pseudomonas fluorescens* and (d) *Rhizobium leguminosarum.*

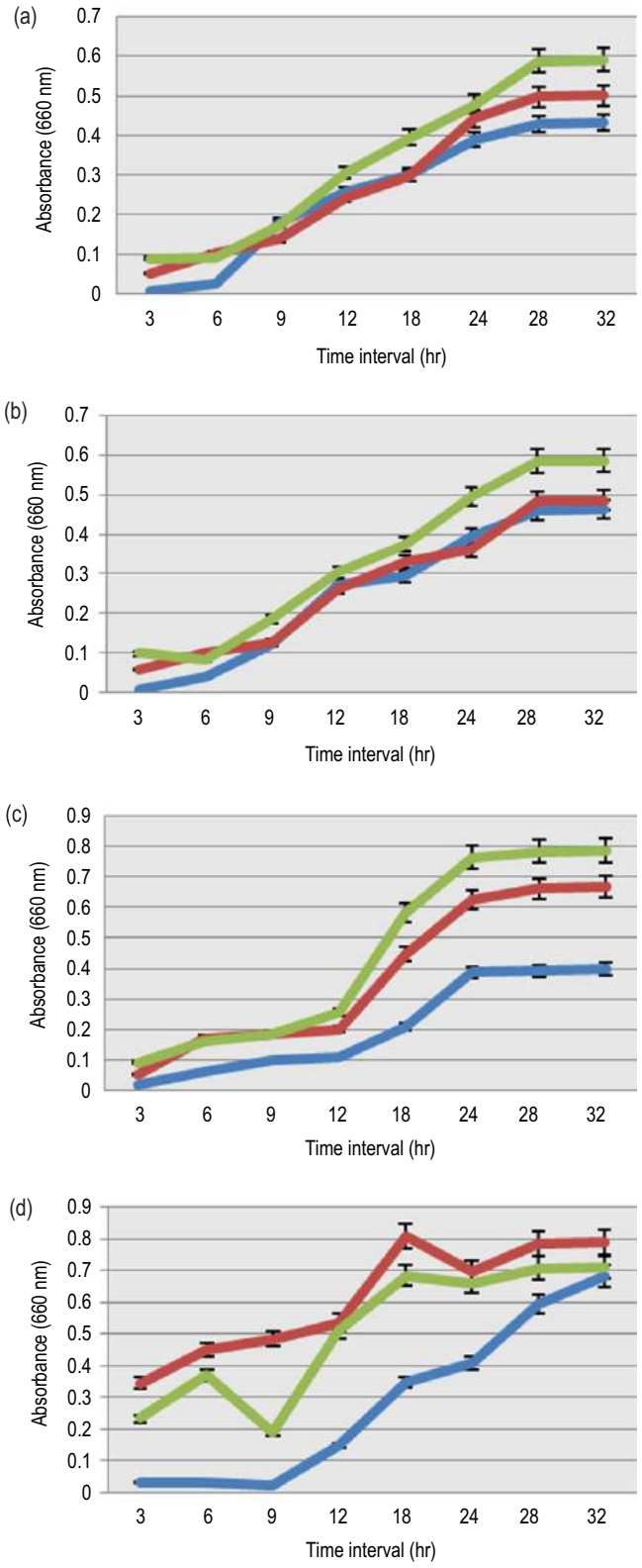

Fig. 3: Growth dynamics of beneficial microorganisms; (a) *Azotobacter chroococcum;* (b) *Bacillus megaterium;* (c) *Pseudomonas fluorescens* and (d) *Rhizobium leguminosarum* treated with zeolites.

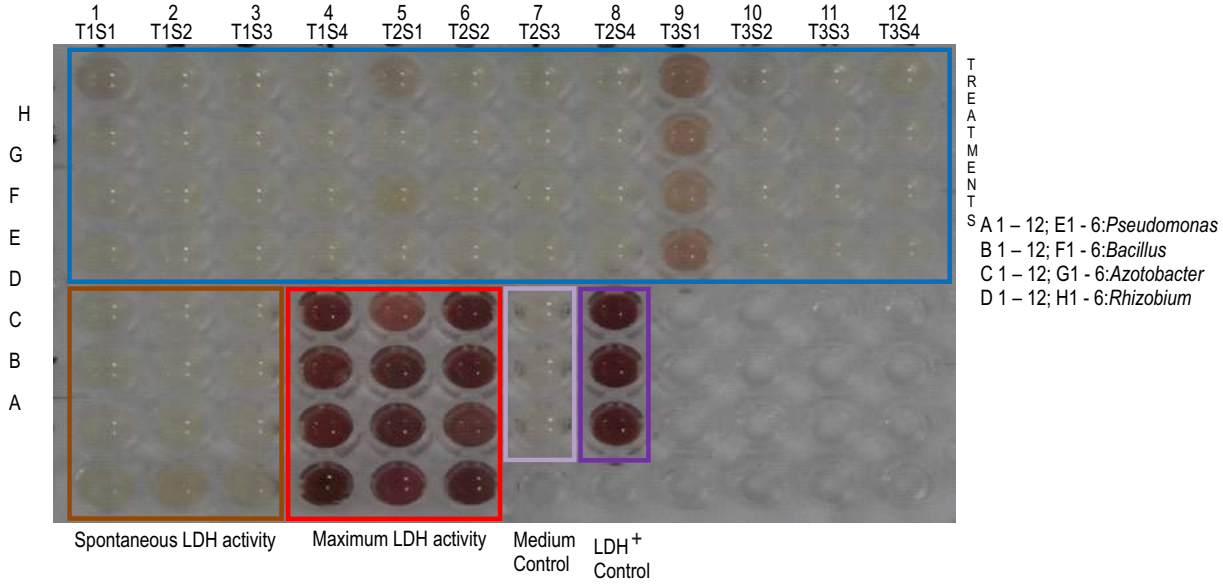

Fig. 4: Assessment of cytotoxicity of zeolite (nano and natural) on beneficial microorganisms using LDH assay.

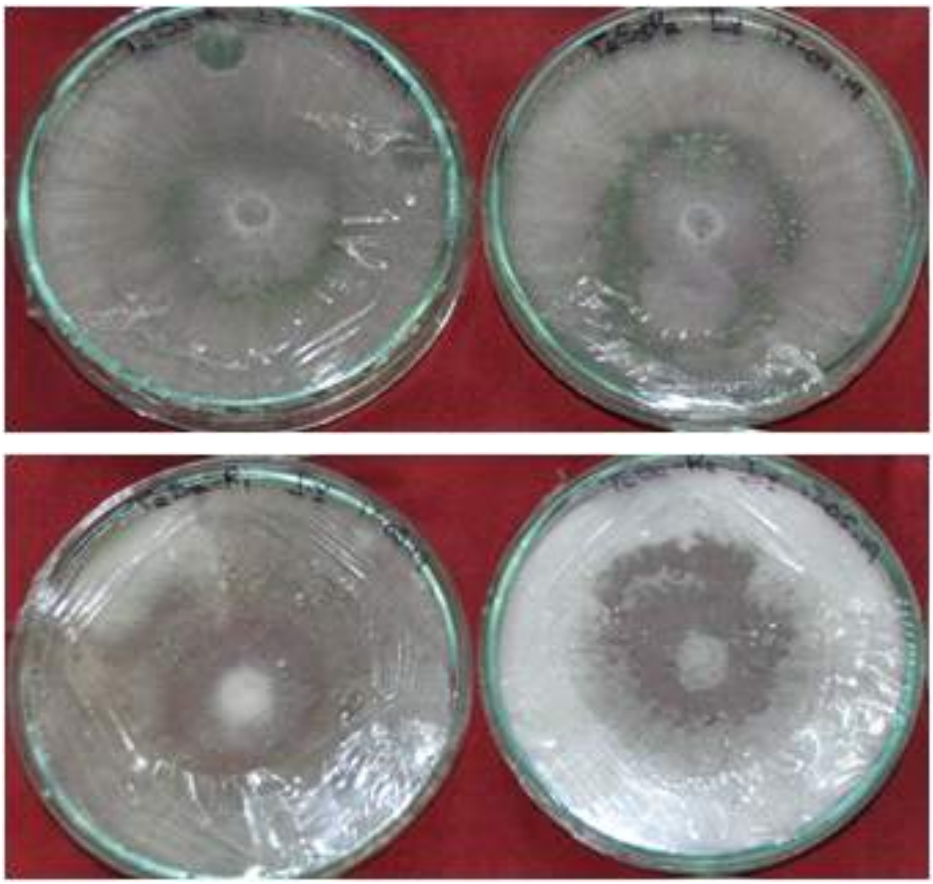

Fig. 5: Zeolites of size regime 500-1000 nm and 200-400 nm showing rapid sporulation.

The data suggest that poison plate technique could not account for the dead cells that might have been generated as an initial exposure of nano-zeolites. This may be attributed to the fact that live cells unexposed to nano-zeolites in the nutrient medium shall mask the cytotoxic effects. Such observation is supported with our data on genotoxic assay in which the micro slides depicted that there was no quantifiable DNA damage in nano zeolite treated cells as compared to the control cultures. These results corroborates with the recent findings of Wu *et al*., (2020) who reported zeolite enriched compost showed enhanced microbial growth indicating that these materials have no toxic effects.

The Average Linear Growth Rate of *Trichoderma viride* was recorded by measuring the average of three diameters and the results are presented in Table.3. In general, incremental dose of zeolites recorded significantly highest linear growth when compared with control. Zeolites at 2000 ppm recorded the maximum ALGR and the lowest in control. Among different sizes of zeolites, 500-1000nm (S2), 200-400 nm (S3), 1000-2000 nm (S4) recorded significantly higher ALGR (22.8, 23.07 and 22.44 mm per day) compared with control (21.50 mm per day) whereas nano-zeolite (S1) treated ones were not significantly higher with a mean value of 21.74 mm per day. The data indicated that incremental concentration of zeolite had a positive effect. In addition, it was also observed that the zeolites in the size regime of 500-1000 nm and 200-400 nm promoted rapid sporulation. Our data is in conformity with the previous studies (Badalova 2014; Kivanc, 2005; Sayıt Sargin, 2013) where zeolites promoted hyphal growth and triggered rapid sporulation as a result of better cellulose activity in the medium.

Overall, this study unequivocally demonstrated that zeolites of size greater than 200-400 nm irrespective of concentration upto 2000 ppm were quite safe for soil beneficial microbes. Though biosafety data of nano-zeolites (1-100 nm) showed enhanced microbial population and increased biomass irrespective of the concentration through poison food technique, LDH assay suggested marginal cytotoxic effects at 2000 ppm, besides Comet assay indicated no genotoxic effects. Hence, zeolite based nanoproducts (below 100 nm) should necessarily undergo high throughput screening and advanced biosafety studies with wide array of biological systems to provide conclusive biosafety information to ensure environmental health.

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Add-on Information

Authors' contribution: L. Sivashankari: Student thesis work. Carried out all the laboratory experiments; **S.K. Rajkishore:** As a guide framed methodology and designed the experiments; **A. Lakshmanan:** Advisory member for result interpretation of zeolite effects on microorganisms; **K.S. Subramanian:** Mentor for overall research and result interpretation; **M. Praghadeesh**: : Standardized cytotoxicity and genotoxicity experiments.

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Antibiotic susceptibility of endosymbionts of papaya mealybug *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae)

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Abstract

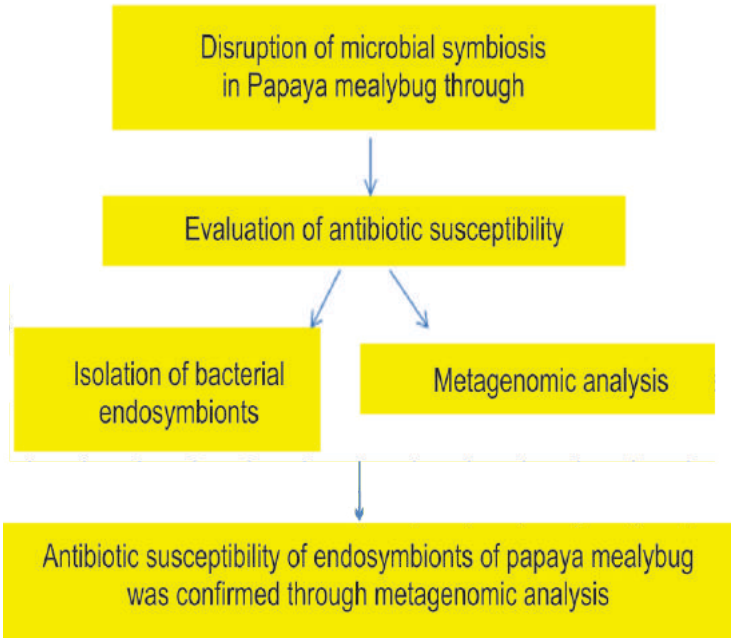
Aim: The present study aimed to select a powerful tool to evaluate the antibiotic susceptibility of endosymbionts of papaya mealybug.

Methodology: Antibiotic Ofloxacin (0.25µg) was used to disrupt the endosymbionts of mealybug. The antibiotic susceptibility of endosymbionts was studied by conventional method; isolation of symbionts in Luria Bertani Agar and Nutrient Agar and also metagenomic analysis was conducted using nanopore sequencing of 16S rRNA gene.

Results: Molecular identification of isolates showed Gram-positive bacteria *i.e., Bacillus cluasii*, *B. altitudinis*, *B. siamensis* and Gram-negative bacteria, *i.e., Serratia marcescens* and *Stenotrophomonas maltophilia*. Metagenomic analysis using 16S amplicon sequencing resulted in identification of *Candidatus Tremblaya Princeps* (55%) was more abundant followed by *Candidatus Mikella endobia* (23%) in untreated mealybug population. Their population was reduced to 7% in *Candidatus Tremblaya Princeps* and below 1% in *Candidatus Mikella endobia*.

Interpretation: The current study confirms that the metagenomic analysis acts as a power lens to evaluate the antibiotic elimination of endosymbionts, hence with this method future works on developing aposymbiotic mealybugs can be done.

Key words: Endosymbiont, Metagenomic analysis, Ofloxacin, Papaya mealybug



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Introduction

The papaya mealybug (PMB) *Paracoccus marginatus* Williams and Granara de Willink (Hemiptera: Pseudococcidae) was recorded for the first time in papaya during July 2008, in Coimbatore district Tamil Nadu. Severe infestation 80 to 90% was observed papaya, mulberry, tapioca, brinjal, tomato, bhendi and flower crops (Muniappan *et al.,* 2008). Throughout their life, papaya mealybug, *P. marginatus* depends on endosymbiotic microorganisms for the supply of essential amino acids and other nutrients, whereby they can live solely on the specialized food source. In the current scenario, the disruption of microbial symbiosis demanded by insect pest and symbionts-mediated manipulation of insect pest traits are the two emerging trends in insect pest control.

Researchers have been unable to isolate the endosymbionts from insect body and culture the same in laboratory conditions. Unfortunately, traditional microbiology and microbial genome sequencing and genomics rely upon cultivated clonal cultures; early environmental gene sequencing cloned specific genes (often the 16S rRNA gene) to produce a profile of microbial diversity in a natural sample. Hence, through traditional microbiological work, the vast majority of microbial biodiversity may miss from documenting. Metagenomics is the study of genetic material recovered directly from environmental samples and use either "shotgun" or PCR directed sequencing to get mostly unbiased samples of all genes from all the members of sampled communities. Because of its ability to reveal the previously hidden diversity of microscopic life, metagenomics offer a powerful lens for viewing the microbial world that has the potential to revolutionize understanding of the entire living world (Schloss and Handelsman, 2005). It appears that papaya mealybug houses number of secondary endosymbionts and many of them are not culturable in laboratory. These facultative secondary endosymbionts may play several roles in its host physiology viz., offers defense towards pathogens and parasites, influence insect- plant interaction, favours the host insect for adaptation to environment, impact on population dynamics and pesticide detoxification.

Materials and Methods

Isolation of culturable endosymbiotic bacteria from papaya mealybug: The culturable endosymbiotic bacteria of antibiotic treated (Ofloxacin @ 0.25µg) and untreated papaya mealybug from papaya host plant were isolated. Second and third instar nymphs (50 numbers) were taken and starved for 6–8 hr to eliminate the bacterial flora acquired through feeding the host plant. The starved nymphs were then surface sterilized with 70% ethanol followed by 0.1% sodium hypochlorite for 30 sec to remove the adhering contaminants, especially external micro-flora. The remnants of disinfectants used for surface sterilization were then cleared by washing thoroughly with distilled water. After final wash, washed distilled water was plated on culture media to ensure complete elimination of external micro-flora. Surface-sterilized nymphs were homogenized using 1 ml of 0.1M phosphate buffer in pestle and mortar.

The homogenates were serially diluted up to 10^{-3}. A 100µl of each dilution of 10^{-1}, 10^{-2} and 10^{-3} were plated separately by pour plate method on two different sterile media such as Luria Bertani agar (10g tryptone, 5g yeast extract, 10g NaCl and15g agar) and Nutrient Agar (5g peptone, 3g beef extract, 5g NaCl and 20g agar) and incubated at 28 ± 2°C for 24h – 72h (de Vries and Visser, 2001). After incubation, the colonies grown on different media were selected on the basis of morphological characteristics such as shape, color and elevation. The selected colonies were subjected to sub-culturing on their respective medium for purification. Five to six subsequent streaking was done to obtain pure bacterial cultures. The purified cultures of endosymbiotic bacteria were maintained by sub-culturing on their respective medium for every 15 days. The purified cultures were examined under a light microscope and stored at -80°C in 50% glycerol.

Molecular identification of bacterial isolates: The bacterial genomic DNA of bacterial cultures obtained from papaya mealybug was isolated using EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt. Ltd., India as per the protocol described in the instruction manual. The isolated DNA was then amplified through PCR (Polymerase Chain Reaction) targeting the 16s ribosomal RNA (rRNA) gene using 27F (forward primer): 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R (reverse primer): 5'-GGTTACCTTGTTACGACTT- 3'. Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using primers. Sequencing reactions were performed using an ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Bio-systems).

Metagenomic analysis of papaya mealybug: About 50, 3rd instar nymphs were taken from each PMB clone and used for metagenomic analysis. Genomic DNA was isolated from the surface sterilized mealybugs using c-TAB and phenol: chloroform extraction method. The isolated DNA was quantified using nanodrop by determining the A260/280 ratio. The endosymbiotic profile of papaya mealybug were studied using Nanopore sequencing platform (Instrument make, Company, Location) with primers 27F 5' AGAGTTTGATCMTG and 1492R 5'AAGGAGGTGATCCAGCCGCA.

Results and Discussion

Molecular identification of isolates showed Gram-positive bacteria, *i.e., Bacillus cluasii*, *B. altitudinis, B. siamensis* and Gram-negative bacteria, *i.e., Serratia marcescens* and *Stenotrophomonas maltophilia* (Table 1). Through conventional isolation and identification only few endosymbionts were isolated and cultured under laboratory conditions. It was not impossible to evaluate the antibiotic

Table 1: Molecular characterization of culturable endosymbiotic bacteria isolated from papaya mealybug

Isolates	Colony morphology	Gram test	Closest match	Genbank accession number
TNAUPS1	White, irregular	+	*Bacillus altitudinis*	Mn907690
TNAUPS2	Cream white, filamentous	+	*Bacillus clausii*	Mn907691
TNAUPS3	Creamy white, translucent	+	*Bacillus siamensis*	Mn907692
TNAUPS4	Red elevated, entire margin	-	*Serratia marcescens*	Mn907693
TNAUPS5	White convex, smooth	-	*Stenotrophomonas maltophilia*	Mn907694

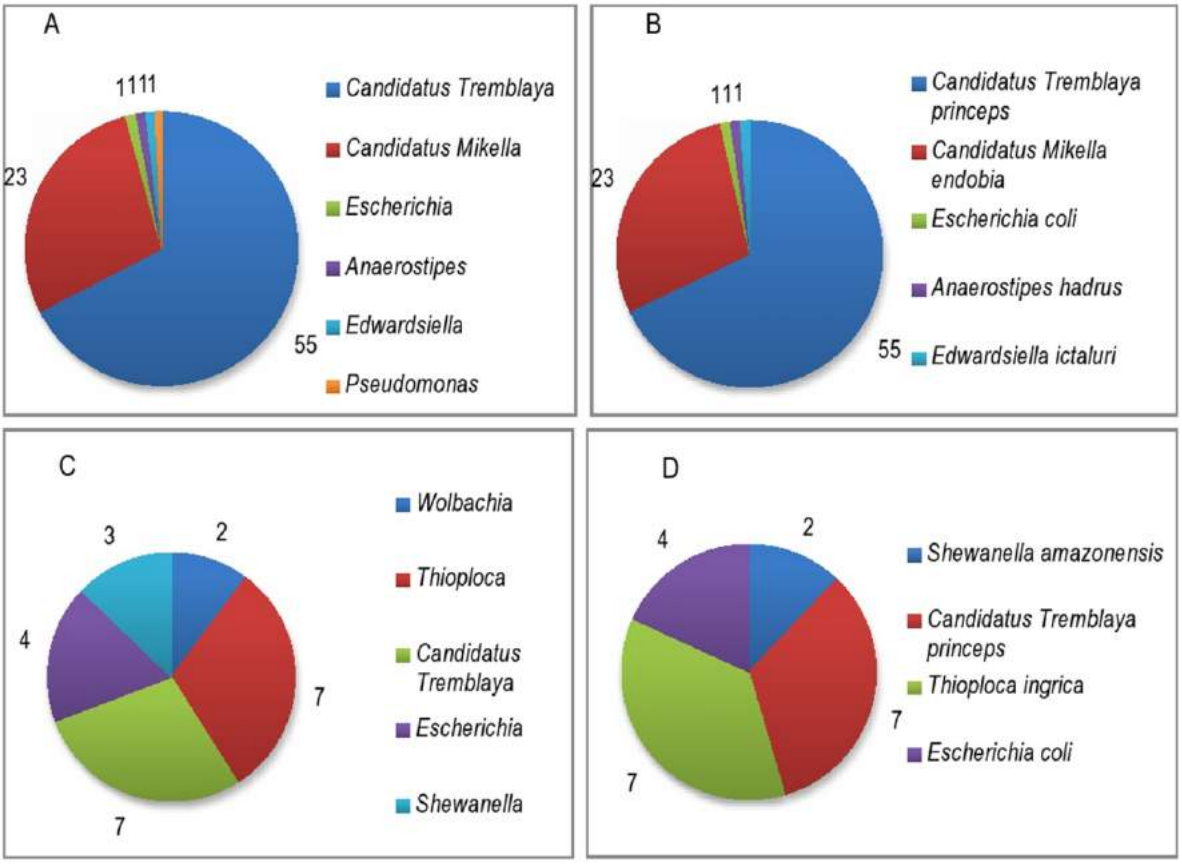

Fig. 1: Per cent abundance of bacterial endosymbionts from papaya mealybugs: (A) At genus level in control mealybug population; (B) At species level in control mealybug population; (C) At genus level in aposymbiotic mealybug population and (D) At species level in aposymbiotic mealybug population.

susceptibility as all the endosymbionts were not cultivable. As there are number uncultivable endosymbionts in insects, hence, metagenomic analysis was performed to explore the whole endosymbiotic profile of Papaya mealybug. Metagenomic analysis indicated that *Candidatus Tremblaya Princeps* (55%) was more abundant followed by *Candidatus Mikella endobia* (23%) in untreated population. *Candidatus Tremblaya* Princeps was reported as the primary endosymbiont of papaya mealybug and its presence is important for the survival and reproduction of its host insect. Their population reduced to 7% in *Candidatus Tremblaya Princeps* and below one per cent in *Candidatus Mikella endobia*.

This clearly confirms the influence of antibiotic ofloxacin on the abundance of endosymbionts of papaya mealybug (Fig. 1). While experimenting the isolation of bacterial endosymbiont associated with mealy bug, Rhizoecus amorphophalli (Hemiptera: Pseuococcidae), three culturable bacteria, namely, *Bacillus subtilis*, *Staphylococcus gallinarum* and *S. saprophyticus* were reported by Sreerag *et al.* (2014). Sreerag *et al.* (2014) reported three culturable bacteria, namely, *Bacillus subtilis*, *Staphylococcus gallinarum* and *S. saprophyticus*. The protective role of *Serratia marcescens* as an extracellular endosymbiont of *Rhynchophorus ferrugineus* was first reported by Scrascia *et al.* (2016). *Stenotrophomonas* sp. has been

recognized to be associated with insects like Collembola. Indiragandhi *et al.* (2007) isolated *Pseudomonas* sp. and *Stenotrophomonas* sp. from the guts of larvae and adults of diamond back moth. Previous studies revealed that different lineages of mealybugs are associated with distinct lineages of bacterial endosymbionts. For example, many species of subfamily Pseudococcinae harbour a beta-proteobacterial endosymbiont Tremblaya princeps and an additional gamma-proteobacterial endosymbiont (McCutcheon and Von Dohlen, 2011; Oliver, *et al.,* 2005; Tamura *et al.,* 2011; Thao, *et al.,* 2002). The primary endosymbionts *T. princeps* are found in almost all Pseudococcine species and exhibit host-symbiont co-speciation (Muniappan *et al.,* 2008; Von Dohlen, *et al.,* 2001). In this study, metagenomics offers access to genetic diversity of uncultivable microbial communities. In future there is a need to explore their metabolic diversity.

In summary, metagenomic data have been useful in understanding the endosymbiotic profile in papaya mealybug with the establishment of both primary and secondary endosymbionts.

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Add-on Information

Authors' contribution: P. Megaladevi: Sample collection, DNA extraction, Molecular Biology, Bioinformatics, Data interpretation, and Manuscript writing; **J.S. Kennedy:** Conceptualization of study, Bioinformatics, Data interpretation, and Manuscript writing.

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Influence of enzymes and extraction conditions on high yield of cottonseed milk

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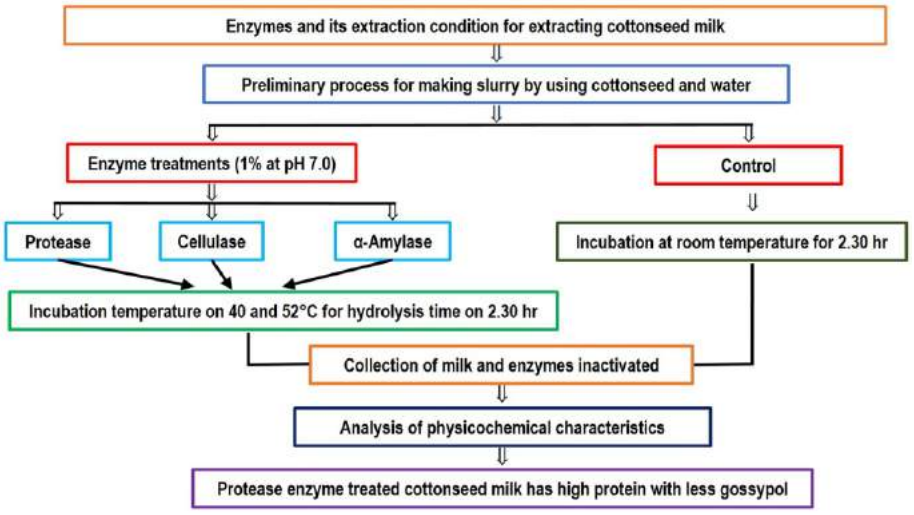
Abstract

Aim: This research aimed to optimize suitable hydrolytic enzymes for maximizing cottonseed milk extracts for high cottonseed milk yield, protein content and low gossypol level.

Methodology: Known amount of cottonseed was soaked for 90 min at 32°C and blended (cottonseed:water@1:6). Different aliquots of the blended cottonseed slurry were treated with 1% of enzymes *viz.,* protease, cellulase and α-amylase enzyme at pH 7.0 followed by incubation at 40 and 52°C for 2.30 hr for the extraction of cottonseed milk. The enzyme activity of extracted milk was subsequently inactivated by pasteurization (90°C, 5 min). Further analysis of physico-chemical characteristics was also carried. The control sample included milk extraction from non-enzyme treated cottonseed milk extract (30±2°C).



Results: Among different treatments, cottonseed milk extraction using protease enzyme at 40°C incubation showed the highest milk yield (86.71%) with the lowest sedimentation (3.72%). Further incubation 40°C and 52°C showed the highest protein content of 2.10 and 2.27 g 100 ml^{-1} and gossypol reduction of 40.36 and 35.22%, respectively, in the cottonseed milk extract. Meanwhile, cellulase and α-amylase enzymes treated samples at both incubation temperatures showed poor physico-chemical characteristics as compared to control.

Interpretation: Protease enzyme seems to be the most suitable for optimum or higher extraction of cottonseed milk.

Key words: Cottonseed milk, Enzymes, Gossypol, Protein

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Introduction

Lactose intolerance is a digestive disorder caused due to the inability to digest lactose in mammalian milk. The digestive enzyme lactase is important for the breakdown of lactose into glucose and galactose. Insufficient production of lactase and/or β-galactosidase at the brush border of human intestine leads to undigested lactose, which moves to the gut system causing gastrointestinal symptoms such as bloating, diarrhea and abdominal pain (Jasielska and Grzybowska-Chlebowczyk, 2019). Consumption of non-dairy food products or avoidance of dairy products is the primary solution for lactose intolerance. As a result of increasing world population, cultural changes, food consumption patterns, veganism and modernization of food and beverage industry, there is immense scope for introduction of lactose free dairy products for the lactose intolerant (Sethi *et al.,* 2016). Hence, plant based milk products are a great alternative for dairy products which also possess the added advantage attributed to inherent functional and nutraceutical components such as fibers, vitamins, minerals and antioxidants for promoting health (Das *et al.,* 2012). Due to increasing demand for non-dairy based food products and growing health concerns, various plant sources are used as alternative for non-dairy milk product.

Suitable plants as non-dairy milk sources include legumes, nuts, seeds, pseudocereals, cereals etc. (Sethi *et al.,* 2016). Among the plant sources, cottonseed is one of the potential source for meeting the requirement properties of milk emulsion with improved nutritional status, but has not been exploited as a source of human nutrition. Cottonseed is a good source of energy, crude protein, fiber content and fat. Also its byproducts have a high biological value and safe fatty acid profile such as 50% monounsaturated fatty acid, 21% polyunsaturated fatty acid and 29% saturated fatty acid which conforms to human health guidelines (Bertrand *et al.,* 2005; Prasad and Blaise, 2020). However, cottonseeds contain toxic compounds such as gossypol, with concentration ranging from 0.02 to 6.64% in seeds among various cotton varieties. The presence of a high amount of gossypol decreases the bioavailability of lysine, arginine and cysteine (Gadelha *et al.,* 2014). Gossypol binds with iron to form gossypol-iron complex, which can affect erythropoiesis and also increase erythrocyte fragility, associated with anemia (Tang *et al.,* 2017). Gossypol associated toxicity issues have been reported in monogastric animals ranging from various degrees of health issues such as loss of appetite, edema of lungs, impaired body weight gain, listlessness and stunted growth.

Studies have shown that gossypol is toxic to monogastric animals, which results in ascites and hepatocyte degeneration in liver, decreased sperm count, spermatogenesis and impaired immunocompetence through reduced number of leukocyte and primarily lymphocytes (Santana *et al.,* 2015; Wang *et al.,* 2019; Zhang *et al.,* 2020). In addition to gossypol toxicity effects, cottonseed has several important phytochemical components of current interest due to the presence of several biological properties, which are used for treatment of leukemia, colon carcinoma, breast cancer, prostate cancer and other malignancies (Xiong *et al.,* 2017; Cao *et al.,* 2018; Yu *et al.,* 2020). The maximum permissible consumption level of gossypol as regulated by the United State Food and Drug Administration (USFDA) is 450 mg kg^{-1} and by the Food and Agricultural Organization/World Health Organization (FAO/WHO) as 600 mg kg^{-1} in cotton seed-based food products for human consumption (Prasad and Blaise, 2020). Normally, plant milks are prepared by crushing the plant material with water followed by extraction of water soluble components.

Extraction of beneficial nutrients such as lipids, flavonoids and alkaloids with water soluble protein components is a complex mechanism, which depends on the chemistry of inner and outer part of cell-matrix, particularly the presence of dietary fiber and other insoluble components of plant material (Sethi *et al.,* 2016). Hydrolytic enzymes such as cellulase, hemicellulase, amylase, lipase and protease, in addition play a major role in extracting these components by cellular fragmentation and destruction, also increases the yield of extracted compounds by modifying the chemical characteristics with improved efficacy (Kirk *et al.,* 2002).

Overall, few studies have been conducted on the extraction of plant-based milk by using enzymes and their interaction in various seeds with the exception of cottonseed. In view of the above, this study aimed to evaluate different hydrolytic enzymes for extraction of cottonseed milk at different temperature for high cottonseed milk yield, high protein content and low gossypol levels, which would provide leverage exploiting of cottonseed as plant based milk for safe source of nutrition and an alternative for animal source milk.

Materials and Methods

Raw material: Cottonseed (*Gossypium* spp.) variety MCU 5 was procured from the Department of Cotton, Tamil Nadu Agricultural University, Coimbatore. Enzymes like protease from *Aspergillus oryzae*, cellulase from *Aspergillus niger*, α-amylase from *Aspergillus subtilis* and USP grade gossypol standard were purchased from Sigma Aldrich, USA.

Extraction of cottonseed milk: 100 g of cottonseed was soaked in 600 ml of water for 90 min at 32°C. Soaked cottonseeds were blended for 90 sec in a blender. Cottonseed slurry samples were separately treated with 1% protease, cellulase and α-amylase enzyme at pH 7.0 incubation of 40°C and 52°C and hydrolysis time of 2.30 hr for the entire enzyme treated slurry samples. After hydrolysis, the slurry was filtered through a muslin cloth and the enzymes were inactivated at 90°C for 5 min and the cottonseed milk was packed in 250 ml flask and stored at 4°C for further analysis. The conventional cottonseed milk extraction process was also carried out by following the same procedure at 30±2°C without adding of enzymes and served as control sample (Kumar, 2019).

Extraction yield: The percentage of cottonseed milk yield by enzyme treated and conventional (non-enzymatic) extraction

process was calculated by the equation given below (Varghese and Pare, 2019).

$$\text{Extraction yield (\%)} = \frac{W_1}{W_2} \times 100$$

Where, W_1 is the cottonseed milk and W_2 is the cottonseed milk slurry.

Sedimentation percentage: Sedimentation percentage was measured by weighing the solids after removing the liquid through centrifuging at 2500 rpm for 10 min (Kabašinskienė *et al.,* 2015). It was calculated by the following equation:

$$\text{Sedimentation (\%)} = \frac{W_1}{W_2} \times 100$$

Where, W_1 represents solids and W_2 cottonseed milk.

Protein estimation: Protein content was estimated by the Kjeldahl method from the crude nitrogen content and using the conversion factor of Nitrogen x 6.25 to arrive at crude protein content (AOAC, 2019).

Gossypol estimation: The gossypol content of samples was determined by spectrophotometer with slight modification (Sadasivam and Manickam, 2005). A 0.5 ml of phloroglucinol reagent and 1 ml of concentrate hydrochloric acid was added to 0.1 ml of cottonseed milk. It was incubated at room temperature for 30 min with 5 min of occasional shaking condition and made up to 10 ml using 80% ethanol and vortexed for 10 sec. The solution and aliquots of different concentration of gossypol standard *viz.,* 20, 40, 60, 80 and 100 µg ml^{-1} were read at 550nm absorbance in UV-Vis double beam spectrophotometer. A calibration curve was plotted between gossypol concentration and absorbance.

Statistical analysis: Statistical analysis was performed by SPSS 17.0 software for One way analysis of variance. The results are based on the mean value of three analytical values and SD. The significance of difference (p<0.05) was evaluated by the influence of different enzymes and the extraction conditions of cottonseed milk.

Results and Discussion

The physico-chemical characteristics such as extraction yield, sedimentation percentage, protein and gossypol content were assessed in the extracted cottonseed milk treated with different enzymes and incubation conditions. The effect of enzymes and incubation conditions on the extraction of cottonseed milk are presented in Fig. 1. The results indicated that protease and α-amylase enzymes at 40 and 52°C resulted in maximum milk yield of 86.71 and 88.80% as compared with protease enzyme at 52°C (79.04%), cellulase enzyme at 40 and 52°C (75.09 and 80.32%) and α-amylase enzyme at 40°C (84.29%) compared to the control sample milk yield as 88.21%, which was par with protease (40°C), α-amylase (52°C and 40°C) treated samples. The effect of enzymes and its incubation temperature on sedimentation percentage on cottonseed milk extract is presented in Fig. 2. The results revealed higher sedimentation percentage in α-amylase treated samples *viz.,* 18.54 and 19.26% at incubation temperatures of 40 and 52°C. Comparatively, less sedimentation percentage was observed in control (2.54%) followed by protease enzyme treated samples (3.72%) at 40°C and 3.52% at 52°C incubation temperature and cellulase enzyme treated samples on 6.04% at 40°C and 7.32% at 52°C incubation of the extracted cottonseed milk.

It was found that the concentration of α-amylase enzyme and incubation conditions were more effective in starch breakdown and hindered the protein matrix, which disturbed cell wall integrity and enabled to increase the extractability of sugars in plant sources (Gebremariam *et al.,* 2013). The starch molecules tend to sediment quickly due to gravitation, and higher molecular size than protein molecules (Bertoft, 2017). Carbohydrase enzymes confer improved digestibility by breakdown of cellulose, hemicellulose and pectin fractions of cells to small oligomers and monomers (Marsman, 1997). The protein and gossypol content of extracted cottonseed milk as influenced by the application of different enzymes and incubation conditions compared to non-enzymatically treated control samples are presented in Table 1. Among the different samples, the protease enzyme treated samples at the incubation condition of 40 and 52°C showed significantly higher (p<0.05) in protein content of 2.10 and 2.27 g 100 ml^{-1}, respectively compared to highest protein in control (2.40 g 100 ml^{-1}) and lowest in α-amylase at 52°C treated sample (1.18 g 100 ml^{-1}).

The cellulase enzyme treated samples at 40 and 52°C showed 1.66 and 1.31 g 100 ml^{-1} of protein in extracted cottonseed milk. Proteolysis increases the protein solubility as well as reduces the anti-nutritional factors due to reaction of protease and also has positive effects on the extraction of protein and oil yield by the combined usage of cellulase and protease

Table 1: Effect of enzymes on chemical characteristics of extracted cottonseed milk

Treatments		Chemical characteristics	
		Protein (g 100 ml^{-1})	Gossypol (mg 100 ml^{-1})
Control		2.40±0.10^{a}	15.56±0.38^{e}
Protease	40°C	2.10±0.01^{c}	9.28±0.38^{a}
	52°C	2.27±0.02^{b}	10.08±0.44^{b}
Cellulase	40°C	1.66±0.02^{d}	11.36±0.05^{c}
	52°C	1.31±0.05^{e}	12.52±0.35^{d}
α-amylase	40°C	1.35±0.01^{e}	14.88±0.52^{e}
	52°C	1.18±0.03^{f}	15.24±0.54^{e}

Each value mentioned as Mean±SD of three replicate analysis (n=3). The values mentioned in superscripts are significantly differed at p<0.05 in the same column

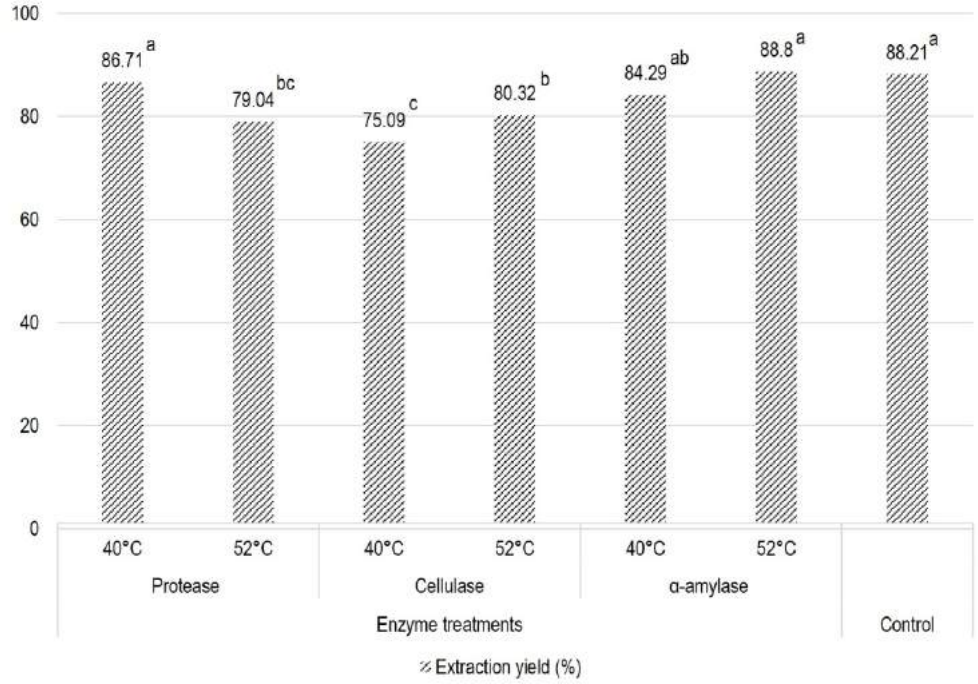

Fig. 1: Effect of enzymes in extracted cottonseed milk yield. Values are mean of three replicate ±S.D. The values mentioned in superscripts are significantly differed at $p<0.05$ in the same row.

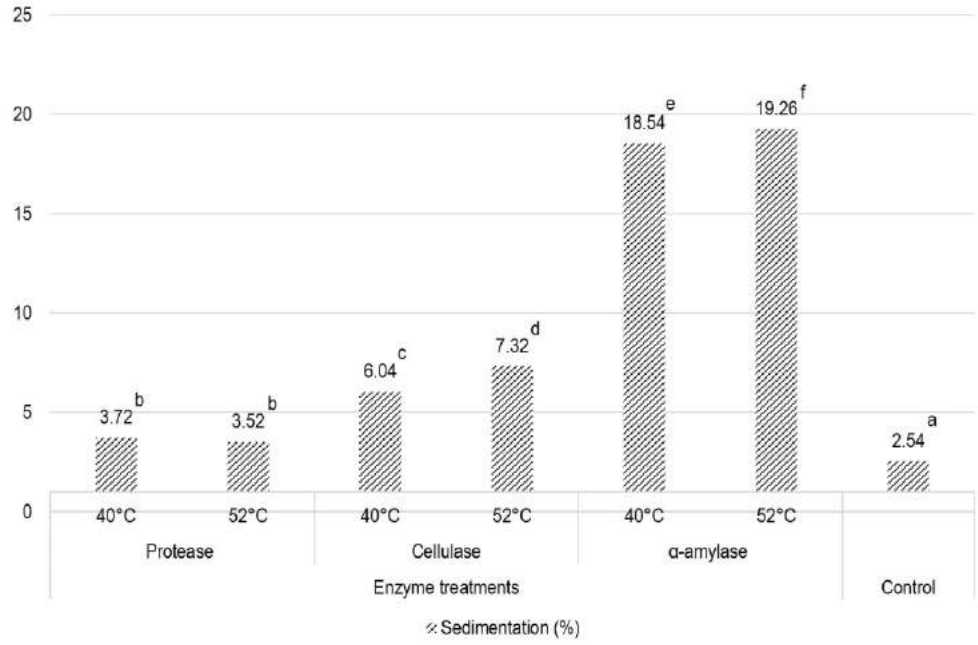

Fig. 2: Effect of enzymes on the sedimentation percentage of extracted cottonseed milk. Values are mean of three replicate ±S.D. The values mentioned in superscripts are significantly differed at $p<0.05$ in the same row.

enzymes under aqueous and solvent extraction conditions (Rosenthal *et al.,* 2001). The relative activity of protease enzyme was 100, 88 and 75% at 40, 50 and 60°C and was also highly active at pH 7.0-9.0 at 40°C in casein hydrolysis (Tang *et al.,* 2010). The presence of protein and oil components in the cell wall suggests that easy release of more protein and oil molecules from the cellular matrix by protein degradation, was achieved by the action of protease enzyme (Passos *et al.,* 2009). Single or combined treatment of protease neutrase and cellulase energex enhanced the protein extractability in soybean meals (Marsman, 1997). Similar findings on protein extractability was observed in extracted cottonseed milk by using protease and cellulase enzymes. The gossypol content of extracted milk was maximum in the control sample (15.56 mg 100 ml^{-1}), which was on par with α-amylase treated samples followed by cellulase treated samples at both incubation temperatures. However, the samples treated with protease enzyme at 40 and 52°C showed 40.36% and 35.22% reduction in gossypol content and exhibited highly significant difference in the control sample.

The plausible reason being that free gossypol renders to modifying the pattern of amino acids released by enzymatic hydrolysis in both structural and functional protein, which results in molecular aggregation and solubility loss of proteins. Chen *et al.* (2019) reported that the total and free gossypol content decreased in the cottonseed meal by adding 2.5 ml of enzyme from *Helicoverba armigera* CYP9A12, at hydrolysis condition of 35°C for 2.5 hr. Whatever, be the selection of cottonseed varieties and hydrolysis condition, such as the concentration of enzymes, pH, time, temperature and microbial sources of enzymes, several factors influence the physico-chemical characteristics of extracted cottonseed milk and its derived products. At room temperature in the exposure of the cottonseed aqueous slurry to prolonged hydrolysis time may be contributing to the growing microbes in the end product.

Both enzymatically treated samples (protease, cellulase and α-amylase) and control samples in addition to the incubation condition resulted in the effective extraction yield, protein and gossypol content of cottonseed milk from the cottonseed with water as the extraction medium. Among the different treatments, cottonseed milk extracted with protease enzyme at 40°C and 52°C showed lower sedimentation and reduced gossypol content with a good amount of protein content and also notable amount of extraction yield. The extracted cottonseed milk meets the permissible intake level of gossypol content as regulated by the USFDA and FAO/WHO. It can be inferred that the cottonseed milk based derived products are more stable than animal based milk products, hence, the lactose free, high protein, low gossypol cottonseed milk may be a suitable alternative for lactose intolerant individuals.

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Add-on Information

Authors' contribution: **S. Thirukkumar:** Carrying this reaserch and preparation of manuscript; **G. Hemalatha:** Providing Food Science lab facility; **S. Vellaikumar:** Providing Biotechnology lab facility; **M. Murugan:** Suggested for statistical analysis; **S. Amutha:** Guided for manuscript preparation.

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