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Temporal variability in foliar protein content and trypsin inhibitory levels in two host trees of tropical Tasar silkworm *Antheraea mylitta*, Drury

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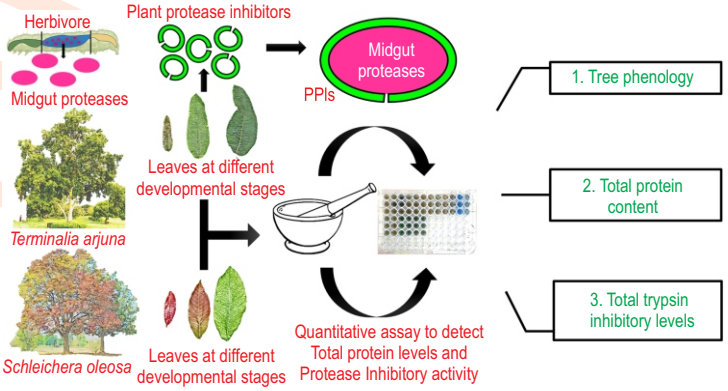
Abstract

Aim: Variation in total soluble proteins and protease inhibitor levels were investigated to determine nutritional and plant defense status in different leaf types of *Terminalia arjuna* (Roxb.) Wight and Arn., and *Schleichera oleosa* (Lou.) Oken, two host tree species of the economically important, non-mulberry Tasar silkworm, *A. mylitta*.

Methodology: Quantitative spectrophotometric assays based on diagnostic amidolytic substrates were conducted to assess serine protease inhibitor activities in young, semi-mature and mature leaf types. A microplate quantification assay for total protein estimations was used with leaf types sampled over a year. Efficacy of total trypsin inhibitor and chymotrypsin inhibitor activities detected in *T. arjuna* (a primary host plant) was further evaluated on gut extracts of fourth instar *A. mylitta* and *Pieris brassicae* L. (a Pierid pest of crucifers) to assess the physiological adaptation of larvae to dietary antifeedants. Molecular provenances with *rbc*L genes were obtained that are available as NCBI accessions #MN460810 for *T. arjuna* and #MT010554 for *S. oleosa*.



Results: Intra-specific variations were evident in leaf phenology of two tree species. Generally, young leaf type of both tree species had high levels of total protein and trypsin inhibitory activities, while mature leaf type of *T. arjuna* had low total protein content and trypsin inhibitor levels. Mature leaf type of *S. oleosa* had low trypsin inhibitor levels during the months of July and August. Midgut proteases of *A. mylitta* and *P. brassicae* were significantly more susceptible ($p \leq 0.05$) to both trypsin and chymotrypsin inhibitors detected in young and semi-mature leaf types of *T. arjuna* than the mature leaf type.

Interpretation: In this study, differential inhibition of digestive proteases in *A. mylitta* and *P. brassicae* by protease inhibitors from different leaf types of *T. arjuna*, suggested adaptation to dietary antifeedants. Such reports on nutritional quality, foliar antifeedants, phenology and host plant utilization are relevant for strategies to domesticate the tropical Tasar silkworm, *A. mylitta*.

Key words: *Antheraea mylitta*, Protease Inhibitors, *Terminalia arjuna*, Total protein, Tree phenology

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Introduction

Tropical Tasar silkworm, *Antheraea mylitta* is polyphagous and can utilize diverse tree species in the tropical monsoon forests of Central India (Barsagade, 2017). Larvae of the economically-important, semi-domesticated 'Daba' eco-race of *A. mylitta* are typically reared on two primary host plants, *T. arjuna* and *Terminalia tomentosa* (Roxb.) Wight. and Arn.,(family Combretaceae) under field conditions. Hence, the outdoor nature of tropical Tasar sericulture poses several challenges en route to realization of high yields for cocoon silk and sustainable livelihood for its predominantly tribal rearers (Barsagade, 2017). According to van Asch and Visser (2007), the host tree phenology and seasonal variation in nutritional quality of diets are important factors that impact ecology of several phytophagous Lepidoptera. Synchrony between insect emergence from diapause and availability of digestible foliage (leaf flush) for larvae is also considered critical for insect performance and survival. With the current scenario of changing climate, phenological studies have regained importance and interest. This is particularly relevant for tropical Tasar sericulture. Bhatia and Yousuf (2014) and Jena (2016) demonstrated that seasonal availability of diet with balanced levels of nutrients is an important prerequisite for rearing this insect.

Ingestion of high-quality foliage from healthy host plants with low impact of dietary antifeedants can contribute to robust growth and development of silkworms along with concomitant increase in cocoon yields. According to Mattson *et al.* (1991), nutritional quality of foliage is known to decline with leaf expansion, due to production and accumulation of secondary metabolites, along with increase in leaf fiber content and reduction in protein content. Dietary protein is an essential macronutrient and differences in protein quantity and/or quality offered by host plants influence patterns of host plant utilization among insect herbivores (Berenbaum, 1995). Dietary proteins frequently include herbivory- induced plant defense proteins such as plant protease inhibitors or PPI (Broadway and Duffey, 1988; Zhu-Salzman and Zeng, 2015). Presence of high levels of PPI is feeding deterrents and causes a decline in quality of total dietary protein. Targets of herbivore-induced PPI are insect gut proteases. PPIs that target specific classes of proteases (serine, threonine, cysteine, aspartic, and metallo- proteases) have been characterized from many plant families and perform diverse functions in regulation of proteolysis (Schuman and Baldwin, 2016). Interaction of herbivory-induced PPI with insect gut proteases is a classical study in insect-plant coevolution and highlights the antifeedant/antinutritive roles of ingested PPI on larval growth and development (Terra and Ferreira, 2012).

In a classical report by Broadway and Duffey (1988),PPIs (like soybean trypsin inhibitor) inhibited insect serine proteases (like trypsin and chymotrypsin) in the alkaline gut of lepidopteran larvae,thereby disrupting digestion of ingested proteins. Physiological responses of larvae to ameliorate negative effects of dietary PPI include over production of gut proteases, hydrolysis of ingested PPI and /or production of inhibitor-insensitive gut proteases (Terra and Ferreira, 2012; Zhu-Salzman and Zeng, 2015). Resulting metabolic costs incurred can have a debilitating effect on the larval growth and development, justifying the role for PPI as a direct defense response in plants against herbivory.

Specialists like the domesticated mulberry silkworm *Bombyx mori* L. are able to evade negative effects of mulberry proteinase inhibitors due to long association with the host plant and adaptation of larval gut proteases to mulberry PPIs (Wang *et al.,* 2017). According to Saikia *et al.* (2011), dietary PPIs from family Lauraceae are implicated in differential expression of gut serine proteases and adaptation of *Antheraea assamensis* (Helfer) larvae feeding on *Persea bombycina* Kosterm. and *Litsea monopetala* Roxb. A Kunitz trypsin inhibitor has been reported by Rai *et al.* (2008) from the leaves of *T. arjuna* of family Combretaceae that, if ingested, may affect digestion in larval *A. mylitta*. To the best of our knowledge, PPI activities have not been reported from *S. oleosa*, a minor host plant for *A. mylitta*. In general, few studies relate phenology of tropical trees with foliar phytochemistry (Donaldson and Lindroth, 2008) and herbivory-related PPIs in preferred leaf types. Herbivores are likely to prefer diets with low levels of foliar PPI, even though a poorly defended tree may attract competitor insects. In contrast, high levels of foliar PPI maybe preferred by adapted, specialist insects that produce gut proteases that are not inhibited by the PPI, albeit with metabolic costs.

In this study, seasonal changes in total protein contents and trypsin inhibition activities were examined for young, semi-mature and mature leaf types of *T. arjuna* and *S. oleosa* sampled over a year.In addition, the extent of inhibition of gut trypsins and chymotrypsins from fourth instar *A. mylitta* and *P. brassicae* by protease inhibitors from different leaf types of *T. arjuna* was examined. The results obtained are discussed in terms of phenology, variation in leaf types, plant defense status and likely impact on silkworm performance and relevance for domestication of *A. mylitta*.

Materials and Methods

Site selection and Sample collection: Three trees of *T. arjuna* (A1, A2, A3 with DBH 6.07cm, 20.22 cm, and 24.25cm respectively) and *S. oleosa* (K1, K2, K3 with DBH 23.47cm, 54.2cm, 50.16cm respectively) were randomly selected at the University of Delhi to monitor total protein content and plant protease inhibitor levels over a period of two years (2017-2019). Molecular provenances based on sequences of rbcL genes were obtained as per Bhardwaj *et al.* (2014). Briefly, genomic DNA was isolated from mature leaf tissues of trees A1 and K1, *rbc*L genes were amplified by PCR, amplicons were cloned into pGEM T cloning vector and sequenced on both strands using universal M13f/r primers. These are available as NCBI GenBank accession numbers MN460810 (*T. arjuna*) and MT010554 (*S. oleosa*). Sample specimens are available in Delhi University Herbarium as vouchers # DUH14497 (*T. arjuna*) and # DUH14496 (*S. oleosa*). Leaves from each tree were sampled monthly and divided into

three major types: young (Y), semi-mature (Sm) and mature (M), based upon the morphological attributes like leaf color, texture and size. Leaves were collected from same node number of different branches.No herbivory was evident in the branches and samples collected. Area of the leaves was determined using a freely available software, ImageJ. Monthly observations were made for each tree with respect to four phenophases: Leaf fall, Leaf flush, Flowering and Fruiting, marked by mass falling off of old leaves, appearance of fresh leaves, flower buds and fruits, respectively, as per Kushwaha and Singh (2005). Other parameters like leaf length were recorded by tagging three leaves in a non-destructive manner on each tree. Youngest leaf at the apex was labelled at the onset of leaf initiation and the length of leaf was recorded using a scale at an interval of two days until maturation. A leaf was considered as mature when no increment in length was observed for at least two consecutive observations. The measurements were pooled to obtain one reading per species per day. Leaf area was compared statistically by One-way ANOVA in SPSS statistical software (version 21, IBM Corporation, NY, USA). Comparison of means for each leaf type was performed by LSD (Least Significant Difference) test at $p \leq 0.05$.

Total protein estimation of plant and insect gut extracts: Sampling of leaves for biochemical analyses were done primarily in August, when leaves of all types (Y, Sm, M) were available. Total soluble protein in plant and fourth instar gut extracts was estimated following Bradford (1976) using Bio-Rad protein assay kit according to the manufacturer's instructions (catalog# 500-0006, Bio-Rad Laboratories, Inc., Hercules, California, USA). Known concentrations of Bovine Serum Albumin (catalog# 500-0007, Bio-Rad Laboratories, Inc.) was used as a standard. Approximately, 10µl of each concentration of BSA and diluted plant/gut extract was added to the microplate in triplicates followed by addition of 200µl of diluted dye reagent. The plate was covered and incubated for 15 min at room temperature. The absorbance was read at 595 nm on a MultiSkanGO plate reader (N10588, MultiskanGO, ThermoFisher Scientific Pvt. Ltd, USA) against a blank containing only extraction buffer with equivalent amount of dye. Total protein content in each sample of plant and gut extract was extrapolated against BSA standard curve. Total protein mg g^{-1} was plotted on a graph.

***In-vitro* assays to determine Protease inhibitory activity in host plants of *A. mylitta*:** To measure trypsin inhibitory levels over one phenological cycle, young, semi-mature and mature leaves (when available) for each of the six trees were collected in the first week of every month. Collected leaf samples (500mg) were homogenized in 1ml of extraction buffer (0.1M Tris-HCl, pH 8.0; 20mM $CaCl_2$). The homogenate was centrifuged in a refrigerated Eppendorf, 5804 R centrifuge at 17,200g for 30 min. Trypsin inhibitor was quantified in the supernatant as per Broadway and Colvin (1992). Briefly, equal volumes of the supernatant were mixed with bovine trypsin solution and incubated for 10 min at room temperature. Subsequently, 1mM substrate N-α Benzoyl-L-Arginine-p-nitroanilide hydrochloride (BApNA, catalog # B-4875, Sigma-Aldrich) was added to the mixture and kept at 37°C for 15 min. The enzymatic reaction was stopped by adding 30% acetic acid and the absorbance of the reaction mixture was read at 410 nm. BApNA specific activity was measured as change in the absorbance per mg total protein of plant extract. Appropriate controls viz. the plant extracts without enzyme were included to account for the colored pigment in the plant extract. Chymotrypsin inhibitory activity was also measured in a similar way using N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPFpNA, catalog # S-7388, Sigma-Aldrich) as a substrate. Furthermore, the gut extracts of fourth instar *A. mylitta* and *P. brassicae* fed on *T. arjuna* and *T. majus*, respectively, were added to the plant extracts as source of enzymes instead of Bovine Trypsin/Chymotrypsin. Assays to measure total Trypsin inhibitory and Chymotrypsin inhibitory activity were then performed as described above. Data were statistically analyzed by One-way ANOVA, LSD for each leaf type using SPSS Statistics version 21 (IBM Corporation, USA).

Results and Discussion

According to Murali and Sukumar (1993), the tropical trees display distinct phenological phases that are affected by climatic variations. In this study, monthly climate data for temperature, humidity, day hours and rainfall were retrieved from an online weather database (www.worldweatheronline.com) and showed similar trends for the study site and a commercial rearing hub of the tropical Tasar silkworm, Banka (Fig. 1a). Trees of both species displayed high levels of intra-specific asynchrony in terms of appearance of leaf flush, leaf fall and appearance of flowers and fruits (Fig. 1b). According to van Asch and Visser (2007), tree phenology can frequently vary between areas, within areas, and even at the scale of adjacent trees of the same species.

Asynchrony has been reported for several tree species from tropical forests in India by Murali and Sukumar (1993) as well as Kushwaha and Singh (2005). In this study, all three trees of *T. arjuna* faced a dry period with very few or no young/fresh leaves in the months of November-March (Fig. 1b). A leaf flush was observed in the months of March-May for both tree species which tends to coincide with the emergence of Tasar moths from cocoons after diapause. The importance of synchronization between availability of high-quality foliage and different developmental stages of insect herbivores has been known for a long time (Feeny, 1970; Ekholm *et al.,* 2020). According to Donaldson and Lindroth (2008), in case of mismatched phenology, lepidopteran larvae tend to opt for well synchronized hosts available in their vicinity.

This scenario may be relevant to forest plantations and Tasar silkworms. Hunter and McNeil (1997) have reported that insects may remain in diapause during unfavorable conditions (such as the dry season) when food plants of good quality are scarce. Leaves of tropical trees show considerable phenotypic and physiological plasticity in response to varying climate (Murali and Sukumar, 1993; Coley and Barone, 1996; Kushwaha and Singh, 2005). For a host tree, leaf types representing different

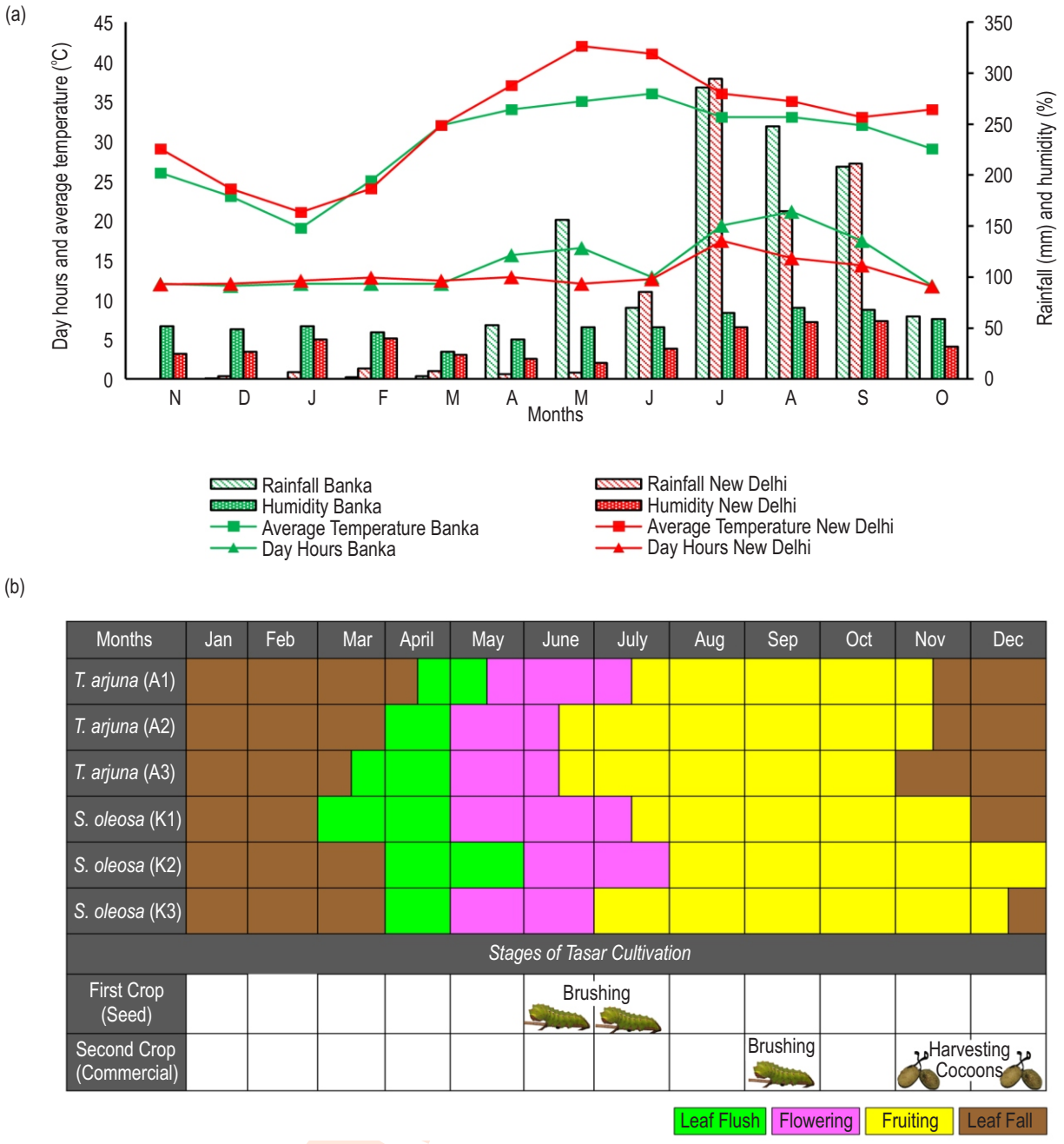

Fig. 1: (a) A typical comparison of the climate at a study site in New Delhi with a Tasar rearing site, Banka, Bihar for rainfall, day hours, average temperature, and humidity (www.worldweatheronline.com) for November 2017-October 2018; (b) Temporal variation in phenological phases (leaf flush, flowering, fruiting, leaf fall defined after Kushwaha and Singh (2005) observed in *T. arjuna* and *S. oleosa*. Major events in rearing of Tasar silkworms are also indicated (Adapted from: https://silks.csb.gov.in/).

developmental stages vary in their attractive on to insect herbivores. According to Brown and Lawton (1991), leaf area (and shape) are also important traits that affect diversity and abundance of phytophagous insects. In this study, the leaf length was measured to assess the growth of leaves from leaf initiation to leaf expansion for both tree species (Fig. 2a). *S. oleosa* leaves were longer as compared to *T. arjuna* leaves and took longer to attain full size. The leaf area was found to be statistically different for young, semi-mature and mature leaves of *S. oleosa* but showed non-significant differences for semi-mature and mature leaves of *T. arjuna* (Fig. 2b). However, the leaves of the latter displayed increase in toughness upon maturity, which is an

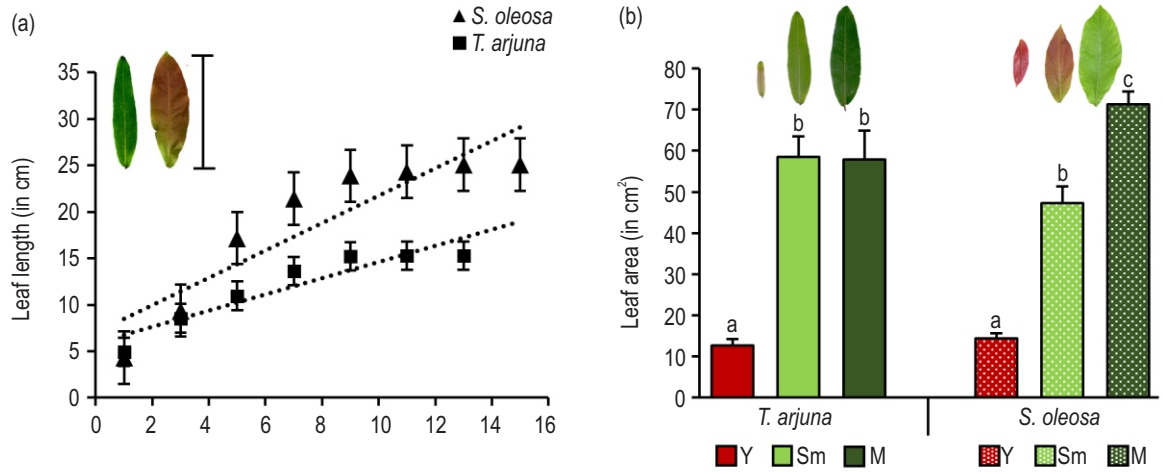

Fig. 2: (a) Leaf lengths of *T. arjuna* and *S. oleosa* in the elongating phase until full expansion. Vertical bars show standard error of average leaf length (n=9) measured on each given day in July-August; (b) Leaf Area of Young (Y), Semi-mature (Sm) and Mature (M) leaves of two host plants. Bars depict mean ± S.E. Significant differences (at p≤0.05, LSD test) are depicted by different letters.

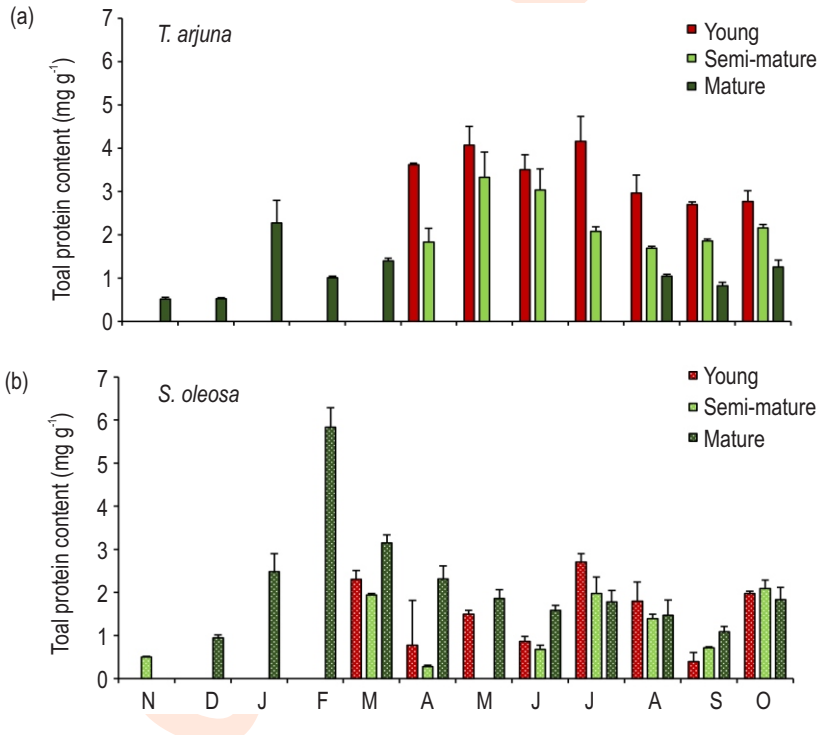

Fig. 3: Annual profile of total foliar protein content in different leaf types of (a) *T. arjuna* and (b) *S. oleosa* (n=9 leaves). Bars depict mean ± S.E.

important defense mechanism of plants against insect herbivores. Softer/younger leaves are generally favored by younger instars of *B. mori* as they have weaker mandibles and are unable to chew tough leaves (Quraiza *et al.,* 2021). According to Despland (2018), asynchrony between host plants and the herbivore affects neonates the most. First and second instars of *A. mylitta* are also reported to be more vulnerable to predator attacks by Gadad *et al.* (2022). Late instars of *B. mori* and other herbivores can chew tough, mature leaves due to well-developed mouth parts (Despland, 2018; Quraiza *et al.,* 2021). Optimal growth of insect herbivores depends on the availability of essential amino acids from dietary protein (Broadway and Duffey,

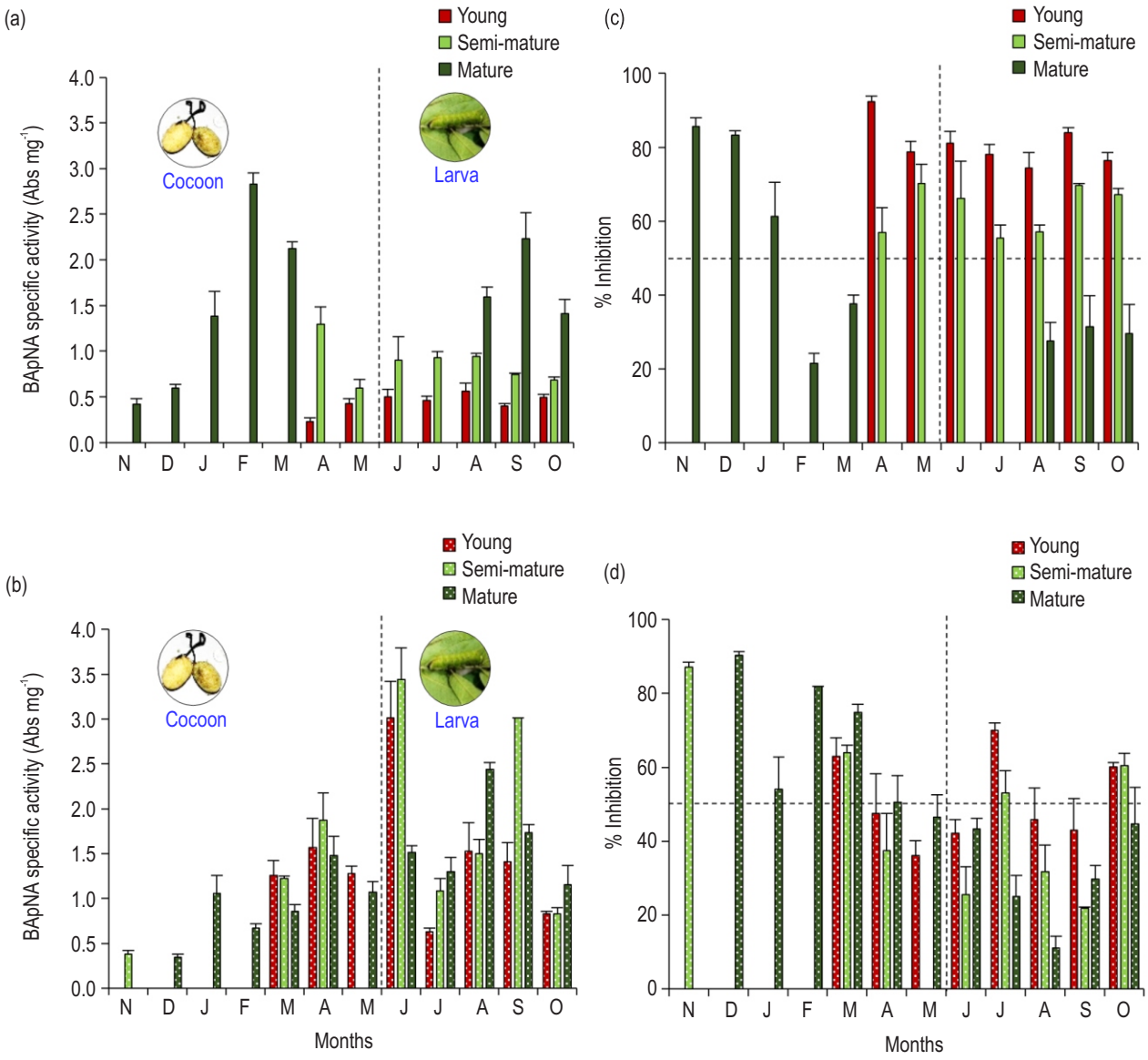

Fig. 4: Annual profile of total foliar trypsin inhibitory activity detected in different leaf types of (a) *T. arjuna* and (b) *S. oleosa* (n=9 leaves). The graphs show amidolytic activity measured against bovine trypsin in three trees of each species. Percentage trypsin inhibitor activities inferred for (c) *T. arjuna* and (d) *S. oleosa* are provided relative to 1mM Bovine Trypsin (taken as 100%). Bars show mean values ± SE.

1988; Berenbaum, 1995). In case of *A. mylitta*, Deka and Kumari (2013) have reported that protein rich diets favor gain in weights of larvae, pupae, and cocoons, as well as enhanced shell weights. In this study, the young leaves of *T. arjuna* had higher levels of total soluble proteins as compared to mature leaves (Fig. 3a). However, no such trend was discernable for different leaf types of *S. oleosa* (under the assay conditions used here), except in July and August (Fig. 3b).

Semi-mature leaves of both tree species contained intermediate levels of total proteins and would be preferred by early-mid instars of the insects. (Fig. 3a) also suggests that leaves available during the first crop (seed crop) were richer in protein as compared to leaves available at the time of commercial rearing. Presence of fewer, nutritionally poor leaves on *T. arjuna* trees during leaf fall season may be implicated in insects remaining in a state of dormancy. According to Bhatia and Yousuf (2014), the availability of host plants with superior food quality can ensure good cocoon harvest in *A. mylitta* and silk yield. For optimal performance, larvae may resort to "compensatory feeding" as described for *B. mori* by Dai *et al.* (2022). According to Bhatia and Yousuf (2014), this behavior may cause increased food consumption, longer feeding time, and extend larval duration during the second crop. Mature leaves with higher leaf area and

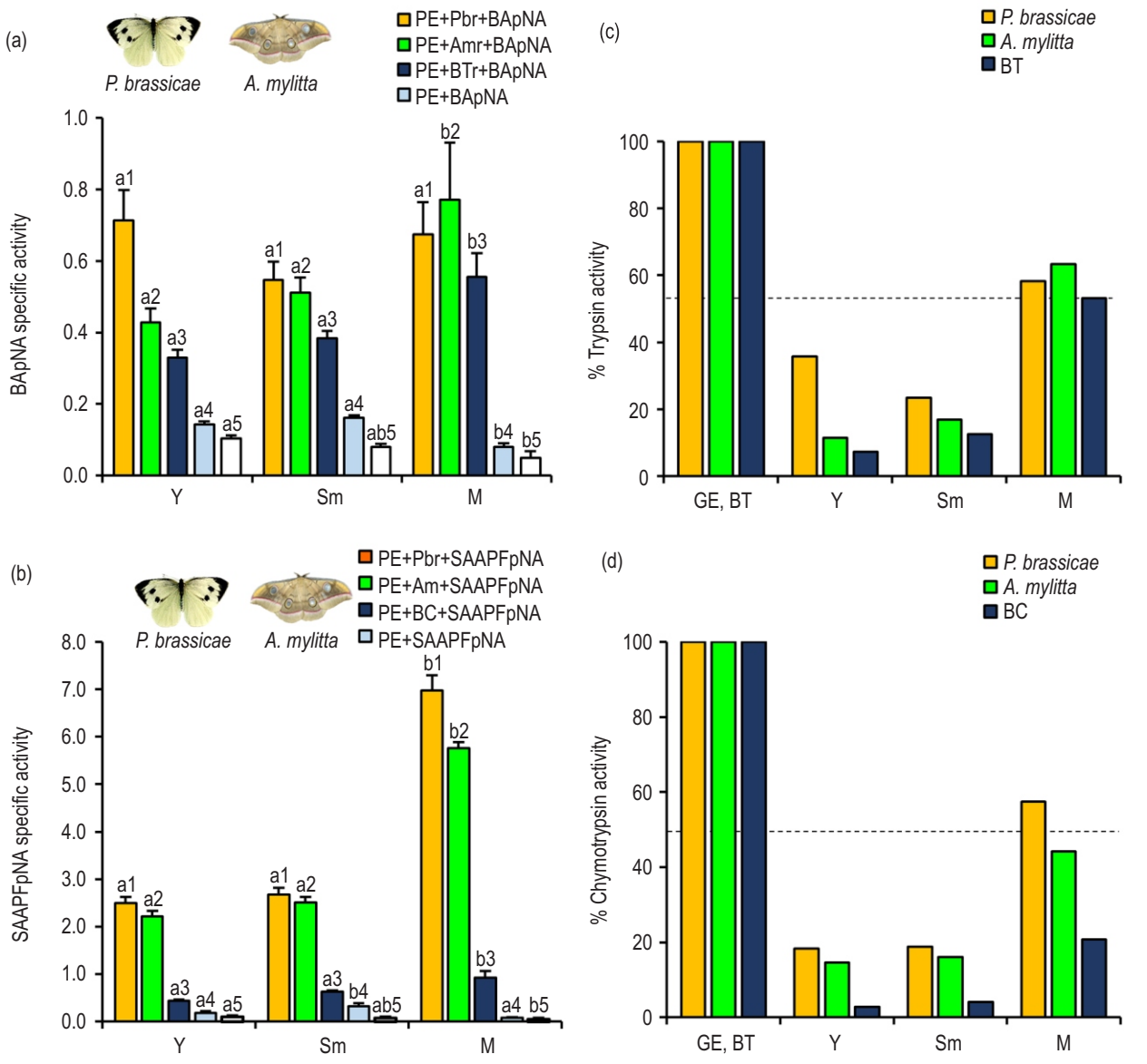

Fig. 5: Estimation of total foliar (a) trypsin inhibitory and (b) chymotrypsin inhibitory activities detected in Y, Sm, and M leaves (n=9 leaves) of *T. arjuna* using gut extract of fourth instar *A. mylitta* (Am, 0.5 µg µl^{-1}) fed on *T. arjuna* and fourth instar *P. brassicae* (Pbr, 0.5µg µl^{-1})fed on *Tropaeolum majus* L., along with Bovine Trypsin (BT, 250ng µl^{-1}) and Chymotrypsin (BC, 50ng µl^{-1}) in August 2019. Bars show mean values ± SE. Controls for only plant extracts (PE) and PE with only substrate (PE+BT/BC) were also included. Significant differences (at p≤0.05, LSD test) for each leaf type are depicted by different letters and numbers. Percent activities remaining for (c) trypsin and (d) chymotrypsin after inhibition by protease inhibitors is shown for each leaf types relative to enzyme activity detected in the absence of inhibitors (taken as 100%).

therefore, higher biomass is available during commercial cycle of silkworm and may offer more food to the insect. Presence of protease inhibitors, tannins, phenolic resins and other antifeedants can reduce nutritional quality and impair herbivore performance in complex ways, including interference with digestibility of ingested plant protein (Broadway and Duffey, 1988; Schuman and Baldwin, 2016). In this year-long study, the monthly levels of total trypsin inhibitory activity were monitored in available leaf types from three trees each of *T. arjuna* and *S. oleosa*. High PPI levels were observed in protein-rich young leaves of both tree species corresponding to low levels of BApNA hydrolysis (Fig. 4). Semi-mature leaves displayed intermediate levels of protease inhibitor activities. This result is consistent with the optimal defense hypothesis. According to the hypothesis proposed by Rhoades (1979), plant defenses are concentrated in the most critical tissues. Furthermore, temporal variation in PPI activities

observed in different leaf types of *T. arjuna* (Fig. 4a,c) indicated upregulation and/or production of PPI-insensitive gut proteases during larval development of Tasar silkworms as an adaptation to corresponding plant defense response in ingested foliage. Metabolic costs of such adaptive responses in *A. mylitta* larvae, if any, have not been estimated. According to Johnson and Zalucki (2007), younger instars of lepidopteran larvae have higher growth rates, metabolic rates, but lower nutritional uptake. This is consistent with Hochuli (2001) who reported limited availability and number of digestive enzymes during early larval development. Point of brushing of neonates and levels of PPI in the host at that phase are thus critical in Tasar rearing activity. A study by Abraham *et al.* (2004) ascribed reduction in growth rate of *A. mylitta* larvae fed on induced foliage of *T. arjuna* to the action of PPIs. In case of results with *S. oleosa*, trypsin hydrolysis levels were generally found to be higher during months of July-September for semi-mature and mature leaves, indicating low PPI levels and high risk of herbivory (Fig. 4b, d).

According to Singh *et al*. (2014), *S. oleosa* trees typically show a wide range of pest infestations during the non-dry period in one phenological cycle. It is also possible that other classes of PPI and/or defense molecules as reviewed by Schuman and Baldwin (2016) provide protection against different insect attackers during the non-dry season in *S. oleosa*. According to Eberl *et al.* (2021), herbivore-specific responses are more cost-effective for a tree than constitutive expression of the same defense response against all herbivores. In this study, BApNA specific activity was measured for *T. arjuna* leaves upon addition of gut extracts of *A. mylitta* and a non-native pest *P. brassicae* (Fig. 5a,c). Amidolytic activity increased on addition of gut extracts indicating low plant trypsin inhibitor (PI) activity. This observation, in turn, implied presence of PPI-insensitive proteases in both insect gut samples. Of all the leaf types tested, semi-mature leaves again showed intermediate levels of PPI activity (Fig. 5a). These results were expected as silkworms are known to perform well on *T. arjuna* trees. The choice of *T. arjuna* as a primary host plant species for commercial rearing of *A. mylitta* larvae has been supported by Bhatia and Yousuf (2014); Jena (2016); Barsagade (2017). Plant defenses in this tree species was expected to be less effective against these larvae. However, comparable levels of PPI activities were also observed with *P. brassicae* gut extracts. These results indicated that gut proteases in *P. brassicae* were insensitive to the inhibitors of *T. arjuna* host plants. Similar trends were observed for plant chymotrypsin inhibitory activities (Fig. 5b, d).

The chymotrypsin inhibitors activities in mature leaves had little effect on the larval gut proteases of both *A. mylitta* and *P. brassicae* (Fig. 5c, d). At the concentrations tested, the insect gut proteases were clearly adapted to the mature leaf chymotrypsin inhibitors in comparison to the bovine protease. The lowest PPI activity was observed in case of mature leaves against gut extracts of both insects and bovine proteases. Further work is necessary on the biochemical and molecular aspects of herbivory-induced plant protease inhibitors in developing foliage of host trees and their interactions with larval gut proteases for downstream applications to improve nutrition for *A. mylitta* and retard digestion in PPI-susceptible agricultural pests. According to Reddy *et al.* (2021), the pest management in Tasar host plants remains a challenge. These trees are either present naturally in forests or grown in plantations for commercial production. Plantations lack diversity and are more prone to native pest attacks that can damage the foliage quality and ultimately affect silk yield. Carefully screened plant protease inhibitors genes can act as candidates for transgenic approaches against native and non-native pests. Production of plant proteinase inhibitors to disrupt larval digestion in an attacking herbivore can be effective against gut proteases of unrelated insects, as suggested for pests of agricultural crops like *P. brassicae* by Kumar *et al.* (2015).

However, Kumar *et al.* (2021) have shown that expression of PPI-adapted proteinases may also occur in the larvae of lepidopteran pest species like *P. brassicae*, limiting the utility of PPI for plant defense. More work from Tasar ecosystem in terms of higher number of samples, and longer study duration is necessary to fully investigate the effects of synchrony between host trees, plant protein and defense levels, and herbivore fitness. Like *B. mori*, domestication of economically-important, non-mulberry silkworms is an important goal in Vanya sericulture. Identification of nutritious foliar feed with unique phytochemistry from diverse host tree species as well as impact of changes in phenology and climates will help in the endeavor to domesticate *A. mylitta*. In addition to potential jump in yields of cocoon silks and benefit to rearers, the goal of domestication will benefit *in situ* conservation efforts for wild populations of *A. mylitta* eco-races that dwell in forests.

The results presented in this paper provide an insight on the underlying biochemical versatility linked to phenological changes in foliage of host plants of Tasar silkworm and the mutual adaptive responses of the silkworm with herbivory-induced responses in the host plant. Understanding these adaptive responses has bearing on better management practices in rearing Tasar silkworms on preferred host plants leading to enhanced silk yield, and also paving the way for its domestication.

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Authors' contribution: P. Bhardwaj Research planning, conducting experiments, interpretation, manuscript writing; **V.K. Choudhary:** Research materials, data interpretation; **M.S. Alam, S. Acharyya:** Research materials, project logistics; **S. Mazumdar-Leighton:** Conceptualization, research planning, supervision, interpretation, manuscript editing; **C.R. Babu:**

Conceptualization, data interpretation, manuscript editing.

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