Assessment of shade-unshade condition and subsequently pesticide treatment on first flush tea leaf metabolites through GC/MS based metabolomics approach

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Abstract: All around the world, tea is one of the most popularly consumed non-alcoholic beverages. Tea is a shade loving shrub. However, how the tea metabolites get changed under shade versus unshade condition and also under shade-pesticide treatment versus unshade-pesticide treatment condition have not been determined and reported yet. So, a metabolomics approach was applied to investigate the shade and pesticide treatment condition of the first flushes of *Camellia sinensis* L. A GC/MS-based metabolomics approach was established encompassing a homogeneous experimental setup for growth, treatment and sampling of tea leaves and their subsequent data analysis using statistical and chemometric tool. A total of 90 metabolites have been identified and semi-quantified. The chemometric analysis elucidates the changes in important metabolites under shade and unshade conditions and also the effect of thiamethoxam on metabolite profile and

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PUBLIC INTEREST STATEMENT

Tea is the most popularly consumed beverage worldwide. Tea was introduced in India by the British as the common man’s drink. Tea industry is one of the oldest industries in India and the teas of India are well appreciated for their unique aromas, flavors, and biological activities. It is well-known that tea has great antioxidant potentiality. The goal of this study is to provide an insight into the changes in metabolic network and antioxidant capacities of first flush tea samples collected from tea garden of North Bengal University campus, exposed to different conditions, namely, shade–unshade (control), shade–unshade (thiamethoxam treatments). Gas chromatography/mass spectrometry-based metabolomic-chemometric approaches identified the distinct changes in chemical composition when exposed to shade than in unshade treatment. Pesticide (Thiamethoxam 25% WG) treatment on shade tea samples showed a decrease in metabolites like important phenolics and organic acids which impart the major flavour, taste, aroma, and superiority in quality of tea and increase in amino acids is also notable.
their responses on tea bushes from shade and unshade conditions. The antioxidant activity and total antioxidant capacity were also assessed and showed a significant change in activity after pesticide treatment both under shade–unshade conditions. Increase in phenols, amino acids, organic acids, sugar alcohols concentrations when plants exposed to shade than unshade treatment was reported. The results suggest that during pesticide treatment on shaded tea plants there is decrease in many important metabolites (phenolics and organic acids), which impart the major flavor, taste, aroma, and quality of the beverage. The increase in amino acids cope the plants to protect them from chemical pesticide treatments under shade conditions.

**Subjects:** Agriculture & Environmental Sciences; Botany; Food Chemistry

**Keywords:** Metabolomics; first flush; shade-unshade; antioxidant; Thiamethoxam; GC/MS; chemometrics

1. **Introduction**

Tea (*Camellia sinensis* (L.) Kuntze) is the most consumed beverage around the world, principally produced and processed from the top two fresh green leaves and a bud. It contains a wide array of important metabolites (such as tannins, alkaloids, terpenoids, flavonoids and polyphenols), which impart its taste, aroma, flavor, and quality.

Tea is a shade loving, perennial, evergreen shrub. This plant is usually grown as a monocrop, under the covering of shade trees. Various metabolism of tea plants are facilitated by shade conditions, which may influence its vigor, superiority in quality, and yield of the beverage crop (Beddage & Mohatti, 2005). As tea is a characteristic subtropical plant, warm, humid atmosphere, acid soil and shade are required for the growth of tea bushes. An ideal shade formed by the trees is a single-covered foliage canopy, which cuts out a large amount of the infra-red rays but allows adequate visible light to infiltrate through the tea bush and does not compete with the tea for nutrients, nourishments, and soil moisture. Some species of *Albizia* such as *Albizia chinensis* (Osbeck) Merr., *Albizia procera* (Roxb.) Benth., *Albizia odoratissima* (L.f)Benth., *Albizia lebbeck* (L.) Benth, *Acacia lenticularis* Buch.-Ham. ex Benth, *Derris robusta* (Roxb. ex DC.) Benth, *Dalbergia sericea* G. Don, *Indigofera teysmanni*, *Cassia siamea* Lam., *Melia azedarach* L., *Dalbergia sissoo* Roxb., *Dalbergia assamica* Benth., *Acacia auricilformis* A.Cunn. ex Benth. and many others are some common shade trees for shading tea bushes (Barua, 2007).

Planting of shade trees has all along been associated with tea cultivation. Shade trees simulate forest conditions. Plants at high altitude are subjected to enhanced oxidative stress due to high UV influence (resulting from air rarefaction, i.e. reduction in density of air) compared to places at low altitude (Balakrishnan et al., 2005; Lesser, 1996). For survival, plants have developed specific cell defensive metabolites, namely, the flavonoids. Flavonoid derivatives (viz. flavonoid glycosides) may also serve as precursor of tea aroma producing compounds and are thus relevant in studies on plant quality under varying UV influence (Bhattacharya & Sen-Mandi, 2011).

Conversely, an important characteristic of shade is its influence on the development of diseases and pests. Mites are normally adverse to shade, but blister blight and black rot diseases increase well in shade. Red spider and other tea mites thrive well under unshade condition (Das, 1959; Banerjee, 1979). Mite damage is more rigorous on unshade rather than shade tea.

So, control of the tea pest is a major challenge in the management of tea plantations. 1,034 arthropods have been identified in tea plantations, around the world (Ye et al., 2014). Throughout the major tea-producing regions, almost 3% of arthropods are common pests, for instance, *Ectropis obliqua*, *Empoa scannonikii*, *Euproctis pseudoconspersa* and so on. Outbreaks of these...
pests account for serious yield losses and decline in tea quality. Different pest control methods are employed globally, such as chemical pesticides, sex pheromones, entomo-pathogenic insect pesticides, and many others (Ye et al., 2014). The application of chemical pesticides is the prime method used by farmers to control tea pests. However, extreme uses of chemical pesticides to control pests have serious unpleasant effect to the environment, resulting in ecological harm and reduced biodiversity. Pesticide residues also show negative impact regarding food safety and security and ultimately resulting in various health issues (Chen et al., 2017).

In the current scientific scenario, the development of metabolomics has provided a new measurement in the subject of multi-dimensional biology for the comprehensive study of the global metabolic networks (Allwood et al., 2008; Dunn, 2008; Krishnan, 2005; Spratlin et al., 2009; Vinayavekhin et al., 2010). Metabolomics has been defined as the comprehensive qualitative and quantitative profiling of a large number of metabolites of a biological system (Fiehn et al., 2000; Trethewey et al., 1999). The major advantage of metabolomics is the simultaneous monitoring of metabolic networks in a way that enables the association of changes in such networks with biotic and/or abiotic causal agents and the detection of corresponding markers.

Tea plant metabolites are altered through the influence of environmental factors such as insect attacks (Kessler & Baldwin, 2002; Paré & Tumlinson, 1999), differential shading treatment (Yang et al., 2012), light quality (Fu et al., 2015), and temperature (Katsuno et al., 2014; Zeng et al., 2016; Zhou et al., 2017). Present study has been designed to evaluate the effect of shading and unshading treatment on the first flush of tea leaf metabolites. In addition to this, to determine the changes in the quality and quantity of metabolome composition under thiamethoxam (25% WG) treatment for the management of tea mosquito bug which causes major damage for first flushes, under shade versus unshade condition in comparison with the shade-pesticide-treated and unshade-pesticide-treated tea plants using GC/MS-based metabolomics technology.

Plant constituents form an important source of antioxidants that scavenge free radicals and prevent cell and tissue damage thereafter by terminating the free radical chain reactions (Mruthunjaya et al., 2016). The antioxidant properties of the samples grown under shade, unshade conditions as well as their pesticide-treated samples were also measured spectrophotometrically.

2. Materials & methods

2.1. Collection of tea leaf samples
First flushes from Camellia sinensis (L.) Kuntze (cv. TV26) were collected from the tea garden of University of North Bengal campus. Four tea bushes were grown at the same tea garden of university campus (26.7095° N, 88.3542° E) with temperature ranging in an average maximum of 14.4°C and an average minimum of 6.3°C during the month of March. Two bushes were selected under shade condition under Dalbergia sissoo and the other two bushes were selected under unshade condition. No chemical pesticide was applied to one set of bush under shade and unshade conditions and were selected as control group of shade plants and control group of unshade plants. The other sets of bush distant from the control group, grown under shade and unshade plants were treated with chemical pesticide (Thiamethoxam 25%WG) (0.250 g/100 mL in distilled water) at a permissible dosage per Plant Protection Code (July, 2019, Ver. 11.0). The soil type, fertilization schedule, and other tea plantation management methods were kept same among the plants. The plucking of two leaves and one bud from each bush was confined during the first flush in the month of March, 2019.

2.2. Chemicals and reagents
The chemicals: adonitol, MOX, MSTFA, MO (C8), MD (C10), ML (C12), MAME (C14), MP (C16), SAME (C18), MA (C20), MB (C22), MT (C24), MH(C26) were procured from Sigma Aldrich (St. Louis, MO); and pyridine from Merck Specialities Private Limited, India. All the other reagents used for sample preparation were of analytical grade, and all the solvents used for GC/MS were of HPLC grade.
2.3. Experimental design
Maintaining the Tea Board of India guidelines, the tea was plucked (two leaves and one bud) from non-pesticide-treated shade, non-pesticide-treated unshade, pesticide-treated shade and pesticide-treated unshade plant bushes.

3. Sample preparation for GC-MS analysis
After harvesting the first flush, the samples were immediately ground to a fine powder with liquid nitrogen. The freeze ground samples of approximately 100 ± 10 mg were taken in 2 mL eppendorf tubes (x4) and extracted with MeOH (HPLC) and H₂O (HPLC) in a ratio of 1:1 (v/v) at 65°C for 30 min in a boiling water bath. 20 μL of 0.2 mg/mL solution of ribitol (adonitol) was added as internal standard. After extraction, MeOH (HPLC) and H₂O (HPLC) were again added in 1:1 (v/v) ratio, and the extracted sample was cold-centrifuged (REMI C-24) at 14,000 rpm for 20 min. The aliquots were distributed in eppendorf tubes (100 μL x4) and evaporated to dryness. The residue obtained was re-suspended in 5 µL of MOX (20 mg/mL in pyridine) and subsequently agitated for 90 min at 30°C. Thereafter, 45 μL of MSTFA was added and incubated at 37°C with frequent agitation for 30 min for trimethylsilylation to increase the volatility of metabolites. 1 µL of FAME markers (a mixture of IR1 markers) were added [prepared using FAMEs of C₁₀—C₂₆ linear chain length dissolved in Chloroform (HPLC)]. GC/MS analysis (Agilent 7890 A GC equipped with 5795 C inert MSD with Triple Axis Detector) was carried out following the method of Kind et al. (2009) with some modifications (Das et al., 2016). Prior to analysis, the samples were preserved at 4°C for 10 min to maintain equilibration and sedimentation of particles.

3.1. Temperature program for GC/MS analysis
For separation and detection of analytes, DB-5 MS capillary column (Agilent J&W GCcolumns, USA) (30 m × 0.25 mm × 0.25 μm) was used. Injection was made in sandwich mode with fast plunger speed without viscosity delay or dwell time. The analysis was performed under the following oven temperature program: oven ramp 60°C (1-min hold) to 325°C at 10°C/min, 10-min hold before cooling down, 37.5 min run time. The injection temperature was set at 250°C, the MS transferline at 290°C, and the ion source at 230°C. Helium was used as the carrier gas at a constant flow rate of 2.5 mL/min (carrier linear velocity 57.95 cm/s). Samples (1 μL) were injected via the split mode (split ratio 1:5) onto the GC column. Prior to analysis, the method was calibrated with the FAME standards available on the Fiehn GC–MS Metabolomics library (2008) (Agilent ChemStation, Agilent Technologies Inc., Wilmington, USA) following users’ guide. AMDIS was used to deconvolute GC–MS results and to identify chromatographic peaks. Auto-tuning of MSD was done at least once a week. All samples were measured in a randomized fashion.

The metabolites were identified by comparing the RT, RI of the metabolites and also by comparing their MS fragmentation patterns of the mass spectra with the entries of compound in Agilent Fiehn GC/MS Metabolomics library (2008) (Agilent Technologies Inc., Wilmington, USA) using metabolite database-Automated Mass Spectral Deconvolution and Identification System (AMDIS) using Agilent RTL method. Retention time of some of the compounds were also compared with that of the standards for confirmation of the metabolites.

4. Determination of antioxidant activity

4.1. DPPH radical scavenging activity
Following the method described by Braca et al. (2001), the antioxidant activity of all the four extracts (unshade control, unshade pesticide treated, shade control and shade pesticide treated) were determined based on the scavenging of the stable DPPH free radical. 0.1 mL of aqueous methanolic leaf crude extracts was added to 3 mL of DPPH solution prepared in methanol in a concentration of 0.004%. After 30 min of incubation, the absorbance was measured at 517 nm wavelength and the percentage inhibition activity was calculated using the formula \(\left[\frac{(A_0-A_e)}{A_0}\right] \times 100\), where \(A_0\) = Absorbance without extract; \(A_e\) = Absorbance with extract.
4.2. Superoxide (O$_2^-$) radical scavenging activity

In the riboflavin-light-NBT (nitro blue tetrazolium) system, superoxide radical scavenging activity was measured with the tea leaf extracts. In a 3 mL volume of total reaction mixture, 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT, and 1 mL of plant sample solution were added (Beauchamp & Fridovich, 1971; Dasgupta & De, 2004). After 10 min of illumination of a fluorescent lamp, the increase in the production of blue formazan was measured at 560 nm.

4.3. TAC

The reaction mixture contained 0.1 mL of tea sample solution and 1 mL of reagent mixtures (mixed phosphate buffer, sulphuric acid, and ammonium heptamolybdate in a ratio of 4:3:3) (Prieto et al., 1999). The reduction of Mo$_{V_2}$ to Mo$_V$ by the extract and the formation of green phosphate/Mo$_V$ complex at acidic pH were assayed. The TAC was measured as equivalent to ascorbic acid.

4.4. Statistical analysis

All the antioxidant assays were performed thrice. From the replicates within the experimental assays, mathematical calculations like means and standard errors were performed by Microsoft Excel 2013. The data were statistically analyzed using t-test of Microsoft Excel 2013 (p-values < 0.05 considered significant).

For chemometric study, the shade versus unshade plants (control), shade versus unshade (treated) and control (shade, unshade) versus treated (shade, unshade) groups were subjected to MVA individually.

Prior to MVA, the missing values were replaced with half of the detection limit (Fiehn, 2006) followed by normalization of the data. The RRRs of all the compounds identified were considered by normalizing the peak areas of the compounds by sample weight in gram and by peak area of the internal standard. The RRRs of metabolites were correlated with the control group of shade plants, control group of unshade plant, shade plant with pesticide treatment, and unshade plant with pesticide treatment. The RRRs were considered as the independent variables and the different treatment (shade, unshade and chemical pesticide—25% thiamethoxam WG) on plants were taken as the modeled responses.

The normalized data were then converted to comma delimited version (.csv) and then subjected to Metaboanalyst v.4.0. The purpose of this study was to determine the differences in metabolite profiling among a set of treatments [shade, unshade (control group) condition and shade, unshade plant treated with chemical pesticide-Thiamethoxam 25% WG (experimental group)] on first flush of Camellia sinensis cv. TV 26 leaves and also to identify the important metabolites responsible for such differences.

So, to determine the changes in metabolite profile pattern, the normalized data set was subjected to Metaboanalyst 4.0 PCA, PLS-DA, s-PLS-DA and OPLS-DA with shade versus unshade group of plants and between shade pesticide treated versus unshade pesticide treated and control (unshade and shade) group versus pesticide treated (unshade and shade) plants based on their metabolite profileas shown in Figure 1.

The important variables responsible during the different treatments on plants were plotted based on their VIP scores (Figure 2).

The normalized data were subjected to ANOVA and a post-hoc test based on Tukey’s HSD test was done to verify the significant variances among the different treatments and determined by p-values lower than 0.05. Based on the generalized logarithm transformed dataset, heatmap clustering of samples as well as metabolites was also developed utilizing squared Euclidean distance and ward linkage.
5. Results

5.1. Metabolite profiling and chemometric study
A total of 90 metabolites have been identified (Table 1) including 14 amino acids, 13 organic acids, 10 fatty acids (2 MUFA and 1 PUFA), 1 inorganic acid, 20 sugar and sugar acids, 3 polyols, 18...
phenols and phenolic acids, 1 flavonoid, 1 anthraquinone and 10 others compounds using Agilent Fiehn Metabolomics Library.

The PCA (figure not given), PLS-DA, and OPLS-DA analyses among the shade versus unshade control samples showed distinct differences between them based on their metabolite profiles as shown in Figure 1.

Important metabolites (top 20 metabolites based on VIP scores) detected by PLS-DA loading plots (Figure 2) for their separation were: D-lyxosylamine, O-acetylsalicylic acid, myristic acid, phloroglucinol, talose, piceatannol, L-valine, heptadecanoic acid, L-glutamic acid, oxalic acid, 2-furoic acid, epicatechin, L-glutamic acid, L-pyroglutamic acid, hydroquinone, glycine, lactobionic acid, norvaline and pipecolic acid.

Shade pesticide treated and unshade pesticide treated tea sample metabolite profiles also showed distinct groups between the two as observed in PLS-DA and OPLS DA 2-D scores plot in Figure 3.
| Metabolites                      | Unshade control | Shade control | Unshade treated | Shade treated |
|---------------------------------|-----------------|---------------|-----------------|---------------|
| **Amino acids**                 |                 |               |                 |               |
| L-alanine                       | 0.228304±0.262253 | 0.212886±0.345394 | 0.068182±0.136314 | 0.336665±0.673297 |
| L-allothreonine                 | 0.163525±0.327026 | 0.10835±0.216641  | 0.12634±0.252628  | 0.459594±0.531144  |
| Aspartic acid                   | 2.554867±1.473309 | 5.272614±6.539845  | 2.650476±0.959753  | 2.61298±1.94009  |
| Beta-alanine                    | nd              | nd             | 0.020244±0.040438  | 0.029263±0.058493  |
| L-glutamic acid                 | 0.413567±0.377723 | 2.502489±2.305432 | 0.631403±1.262754 | 1.623468±3.246903 |
| L-glutamic acid (Dehydrated)    | 6.782672±12.37384 | 55.62342±82.96301 | 30.8979±56.77552 | 84.54689±49.75885 |
| Glycine                         | nd              | 0.134463±0.268868 | nd              | nd             |
| L-mimosine                      | 0.02227±0.044516 | 0.036087±0.072116 | 0.005972±0.011893 | 0.01807±0.036106 |
| Norvaline                       | nd              | 0.049121±0.098184 | nd              | nd             |
| L-proline                       | nd              | 0.041844±0.083629 | nd              | 0.04139±0.052537 |
| L-pyroglutamic acid             | 24.4174±18.48638 | 26.42823±36.63091 | 13.3477±10.58989 | 17.66321±14.61166 |
| L-serine                        | 1.08111±0.358114 | 2.445855±1.424546 | 0.798907±0.713352 | 0.68745±0.473888  |
| L-threonine                     | 1.146199±0.526327 | 2.246669±0.833451 | 0.469889±0.226152 | 0.522129±0.274848 |
| L-Valine                        | 0.025866±0.051713 | 0.098788±0.086625 | nd              | 0.026561±0.05309  |

(Continued)
| Organic acids          | nd   | nd   | 0.02114±0.04223 | 0.045758±0.091482 |
|------------------------|------|------|----------------|------------------|
| Citramalic acid        | nd   | nd   | 0.02114±0.04223 | 0.045758±0.091482 |
| Citric acid            | 74.14552±22.85105 | 79.18722±49.96695 | 119.4344±63.66977 | 33.99402±5.378253 |
| 2-Fumaric acid         | nd   | 0.04768±0.076001 | 0.023027±0.028701 | 0.083879±0.016724 |
| Fumaric acid           | 0.2962±0.36043 | 0.349187±0.313853 | 0.653868±0.981346 | 0.271341±0.259791 |
| Glyceric acid          | 5.910106±3.747782 | 6.807381±4.131732 | 3.318404±3.80883 | 1.184313±1.38211 |
| 2-Isopropylmalic acid  | 0.35755±0.14723 | 0.545956±0.438255 | 0.019841±0.039638 | 0.051153±0.102273 |
| Itaconic acid          | 0.010086±0.020148 | 0.00799±0.015921 | nd               | nd               |
| D-Malic acid           | 24.20463±18.11542 | 24.25882±24.4226 | 18.38013±13.47434 | 16.97043±2.835745 |
| Malonic acid           | 0.832814±0.843786 | 0.770864±0.53866 | 0.730688±0.453209 | 0.463886±0.526759 |
| Oxalic acid            | 2.852986±2.19269 | 16.37598±19.61302 | 5.71698±4.995632 | 6.968063±1.810311 |
| Phosphoric acid        | 31.06948±8.176692 | 75.9026±61.99405 | 40.08637±30.82515 | 29.75815±9.056089 |
| Piperolic acid         | nd   | 0.044353±0.088648 | nd               | nd               |
| Succinic acid          | 2.360504±1.746408 | 3.442626±2.864295 | 2.332162±1.407682 | 1.723399±2.343109 |
| Tartaric acid          | 0.820817±1.015132 | 1.454528±1.847278 | 0.14679±0.181551 | nd               |
| Tartaronic acid        | nd   | nd   | nd              | 0.031118±0.062243 |
Table 1. (Continued)

| Sugar and sugar derivatives | D-allose | 51.3131±28.83253 | 123.7945±123.5244 | 92.81678±90.97513 | 101.8083±136.2539 |
|-----------------------------|----------|------------------|------------------|------------------|------------------|
| D(+)-altrose                | 1.265745±2.531471 | 0.739113±1.478209 | nd               | nd               |
| Cellobiose                  | 1.588999±0.698101 | 2.043439±0.451286 | 2.303845±0.715461 | 0.780823±0.763081 |
| Fructose                    | 218.3505±98.30485 | 374.35±333.898 | 375.2242±315.4777 | 172.7313±193.247 |
| Gluconic acid               | 2.140321±1.20238 | 2.79448±1.260453 | 2.058022±1.214619 | 1.440215±1.03126 |
| D-glucose                   | 252.2426±160.7653 | 427.0507±451.2108 | 317.3816±465.7004 | 231.7972±255.034 |
| D-glucose-6-phosphate       | 0.663125±0.46478 | 1.00847±0.925828 | 0.091268±0.182491 | 0.276606±0.32357 |
| Glycerol-1-phosphate        | 2.620493±0.661063 | 6.520595±7.720124 | 2.111053±2.227876 | 1.878857±0.716178 |
| Glycolic acid               | 2.098875±0.719936 | 3.159507±1.942514 | 2.014003±1.257096 | 1.724026±0.964005 |
| L(+)lactic acid             | 2.973021±1.173208 | 5.931806±5.705816 | 3.415905±1.643719 | 1.729651±1.198014 |
| Lactobionic acid            | 1.865037±1.427489 | 4.048182±3.936746 | 2.699823±2.34388 | 0.77558±1.551127 |
| D-lyxose                    | 3.10996±1.944333 | 5.802814±7.603148 | 5.601992±5.52491 | 2.045133±1.633054 |
| D-mannose                   | 45.57689±85.9176 | 188.7942±355.1013 | 49.2431±64.52189 | 13.3727±26.74543 |
| Mucic acid                  | 0.195519±0.2862 | 0.134113±0.167785 | nd               | 0.035496±0.070959 |
| Raffinose                   | 0.6796±1.037192 | 0.71724±0.575397 | 0.259126±0.191291 | 0.15857±0.31711 |
| Ribonic acid gamma lactone | 2.126691±0.529457 | 3.820467±2.50727 | 3.981295±2.1118 | 3.353029±1.810262 |
| D-saccharic acid            | 0.087345±0.17467 | nd               | 0.025835±0.051619 | nd               |
| Sucrose                     | 5.16937±2.73547 | 6.05878±3.249885 | 2.866368±2.196268 | 2.281318±0.514586 |
| Tagatose                    | 177.0988±115.6614 | 503.2685±618.8118 | 297.932±203.021 | 240.3491±208.4497 |
| Talose                      | 46.65011±34.96552 | 12.03628±24.07252 | nd               | 15.6401±13.2802 |
| Sugar alcohols              | Aallo-inositol  | 134.1599±57.5025 | 299.7449±283.4809 | 183.03±91.2323 | 131.55±38.59962 |
| Galactitol                  | 0.726189±1.452354 | nd               | 0.33189±0.663741 | nd               |
| D-mannitol                  | 1.0516±0.243168 | 1.649009±1.633523 | 1.279039±0.672224 | 0.407345±0.281729 |

(Continued)
| Phenolic compounds         | 1                           | 2                              | 3                           | 4                           |
|---------------------------|-----------------------------|--------------------------------|-----------------------------|-----------------------------|
| Phenolic compounds        | 0.294076±0.570375           | 0.593515±1.155436              | 0.015192±0.030333           | 0.020068±0.040103           |
| Benzoic acid              | 0.886347±1.205721           | 0.976571±0.640295              | 0.5086±0.217723             | 0.408284±0.13244            |
| Caffeic acid              | 12.73084±7.138051           | 25.89207±28.88188              | 34.46379±11.01344           | 38.34061±14.28425           |
| Chlorogenic acid          | 7.69020±4.894736            | 7.99137±8.627648               | 34.04217±39.0944            | 16.5838±10.32008            |
| Catechin                  | nd                          | 0.02940±0.058749               | nd                          | nd                          |
| 3,4 dihydroxybenzoic acid | nd                          | nd                             | nd                          | nd                          |
| Epicatechin               | 20.60674±16.46333           | 71.81925±61.180902             | 15.94224±31.88443           | 128.0009±107.6998           |
| (-)-epicatechin           | 19.40894±16.52348           | 56.1947±71.15968               | 55.4804±45.43983            | 249.7252±180.2874           |
| Epigallocatechin          | 9.40675±3.735215            | 38.46769±49.08076              | 26.00509±29.92057           | 169.755±110.2747            |
| Gallic acid               | 413.4861±155.7187           | 922.5808±1116.083              | 957.5726±765.8347           | 280.0642±329.2889           |
| Hydroquinone              | 0.043705±0.05542            | 0.19617±0.160429               | 0.069856±0.034993           | 1.471579±2.891393           |
| 4-hydroxybenzoic acid     | nd                          | 0.023748±0.047442              | nd                          | nd                          |
| 4-isopropylbenzoic acid   | nd                          | nd                             | 0.077038±0.112094           | 0.019326±0.038625           |
| O-acetylsalicylic acid    | 0.667101±0.330096           | 0.088379±0.176705              | nd                          | 0.102518±0.205002           |
| Piceatannol               | nd                          | 4.67746±6.809132               | 1.116735±1.325162           | 0.447503±0.504697           |
| Pyrogallol                | 1.46733±0.16466             | 4.074749±3.031138              | 2.713017±2.467536           | 1.237929±0.654858           |
| Quinic acid               | 3.163701±260.5887           | 731.0759±890.3412              | 581.433±801.7634            | 382.8558±307.939            |
| Shikimic acid             | 8.768288±3.859632           | 11.16984±6.949513              | 11.91889±12.78305           | 3.993225±3.743111           |
| Kaempferol                | 2.52204±0.720207            | 5.074806±5.262654              | 1.029858±1.735029           | 2.842556±0.991512           |
| Alizarin                  | 0.022036±0.044046           | 0.047255±0.060851              | 0.325919±0.387801           | 0.054874±0.109714           |
| Others                     |       |       |       |       |
|---------------------------|-------|-------|-------|-------|
| Adrenaline                | 4.084±2.699 | 3.983±4.446 | 3.889±1.513 | 1.067±1.116 |
| Cytosine                  | 0.023±0.046 | nd      | nd      | nd     |
| 1,3 dihydroxyacetone      | 0.034±0.068 | nd      | nd      | 0.033±0.066 |
| DL-3,4 dihydroxyphenyl glycol | 3.276±2.622 | 5.743±7.078 | 4.288±3.968 | 0.406±0.408 |
| D-lyxosylamine            | nd    | 6.043±9.721 | 5.482±5.909 | 0.781±1.145 |
| Nicotinic acid            | 0.036±0.072 | 0.04±0.046 | 0.014±0.029 | 0.021±0.042 |
| Phloroglucinol            | nd    | 0.280±0.299 | 0.031±0.062 | nd     |
| Porphine                  | 0.226±0.453 | 0.501±1.003 | 0.154±0.309 | 0.236±0.472 |
| Thymine                   | nd    | 0.015±0.031 | 0.004±0.008 | 0.007±0.015 |
| Uracil                    | 0.085±0.106 | 0.074±0.096 | 0.242±0.278 | 0.060±0.079 |
| Urea                      | 0.040±0.081 | nd      | nd      | nd     |
Figure 3. 2-D scores plots of PLS-DA and OPLS-DA analyses showing distinct difference between shade pesticide-treated vs unshade pesticide-treated leaf samples of *Camellia sinensis.*
The VIP scores in PLS-DA (Figure 4) detected the important metabolites between the two groups were: epicatechin, lactobionic acid, kaempferol, tartaric acid, alizarin, raffinose, L-glutamic acid, uracil, L-proline, DL-3,4-dihydroxyphenyl glycol, D-mannose, D-lyxosylamine, shikimic acid, cellobiose, D-allose, oleic acid, talose, galactinol and D-mannitol.

The control group (shade and unshade) and the pesticide-treated group (shade and unshade) also showed clear discrimination between the 4 distinct clusters based on their metabolite fingerprinting as shown in PCA (Figure not given), PLS-DA (Figure not given) and s-PLS-DA 2-D and 3-D scores plot (Figure 5).

The top 20 metabolites were detected as the VIP based on PLS-DA among these four sets of treatments on plant (Figure 6). The top 20 metabolites identified for their separation were: epicatechin, D-glucose-6-phosphate, L-valine, L-glutamic acid, kaempferol, 2-isopropylmalic acid,
Figure 5. S-PLS-DA 2-D and 3-D scores plot among shade control, unshade control, shade, and unshade pesticide-treated leaf samples based on metabolite profile.
phloroglucinol, DL-3,4-dihydroxyphenylglycol, uracil, mucic acid, L-proline, galactinol, D-glucose, 4-isopropylbenzoic acid, D-saccharic acid, catechin, norvaline, piceatannol and alizarin. Differences of the amount of the detected key contributory metabolites (based on VIP scores) were measured as identified by PLS-DA as shown in Figure 6.

The top 10 metabolites identified from s-PLS-DA loadings (Figure 7) were: citric acid, epicatechin, epigallocatechin, kaempferol, L-proline, (+) epicatechin, cellobiose, L-glutamic acid and D-mannitol.
Heatmap (Figure 8) illustrating changes in the relative concentrations of the metabolites between different treatments encoded using a color scale. The two-dimensional hierarchical cluster analyses combined with heat map reveals trends between the different treatments on tea plants and the metabolites (variables).

Overall changes in concentration of the important metabolites during different treatments on tea leaf samples are shown in Figure 9 as developed from VIP scores of PLS-DA (Figure 6).
The TIC of GC/MS analyses of control group of tea bushes under shade and unshade condition and thimethoxam treated tea bushes under shade and unshade condition are shown in Figures 10 and Figure 11, respectively.
Figure 9. Changes in the concentration of top 20 metabolites based on VIP scores of PLS-DA during different treatments on tea leaf samples. The error bars represent standard error of four biological replicates.

Figure 10. Total Ion Chromatogram of GC/MS analysis: A) Control group of tea bushes under unshade condition and B) Control group of tea bushes under shade condition.
5.2. Antioxidant activities

Aqueous methanolic extracts of all the unshade control, unshade pesticide treated, shade control and shade pesticide treated sample extracts scavenged DPPH radical ($IC_{50}$ values being 15.14 µg/mL ± 0.31, 7.26 µg/mL ± 0.20, 8.87 µg/mL ± 0.1515 and 14 µg/mL ± 0.05, respectively) and superoxide radical ($IC_{50}$ values being 18.817 µg/mL ± 0.453, 18.447 µg/mL ± 0.36, 27.293 µg/mL ± 0.064 and 18.597 µg/mL ± 0.408) in a dose-dependent manner. Unshade control sample extract showed highest ascorbic acid equivalent total antioxidant capacity (Table 2).

Table 2. Antioxidant activities of variously treated tea sample extracts

| Tea Extracts (IC$_{50}$ value µg/ml) ± sd | DPPH radical      | Superoxide radical | Total antioxidant capacity |
|------------------------------------------|-------------------|--------------------|----------------------------|
| Unshade (control)                        | 15.14 ± 0.31      | 18.817 ± 0.453     | 0.00042 ± 0.0001           |
| Unshade (pesticide treated)              | 7.26 ± 0.2        | 18.447 ± 0.36      | 0.00036 ± 0.0001           |
| Shade (control)                          | 8.87 ± 0.15       | 27.293 ± 0.064     | 0.00036 ± 0.0002           |
| Shade (pesticide treated)                | 14 ± 0.05         | 18.597 ± 0.408     | 0.00038 ± 0.0002           |
6. Discussion

In this study, a dynamic change in the concentration of metabolites was determined due to shade versus unshade condition on tea plants (Figure 9). Gas chromatography coupled with MS is a powerful tool and a fast and accurate method to determine hundreds of metabolites including sugars, organic acids, polyols, diverse phenolic and cyclic compounds following solvent extraction and derivatization (Rohloff, 2015). Overall, all the phenolic compounds showed increase in response to shade than unshade condition, namely, epicatechin (1.801 fold), kaempferol (1.721 fold), phloroglucinol (14.798 fold), 4-isopropyl benzoic acid (1.165 fold), catechin (0.96 fold), piceatannol (18.815 fold), alizarin (1.1 fold), hydroquinone (2.166 fold), 4-hydroxybenzoic acid (11.193 fold), 3,4 dihydroxy benzoic acid (11.501 fold), benzoic acid (1.013 fold), chlorogenic acid (1.0131 fold), gallic acid (1.157 fold), quinic acid (1.208 fold), pyrogallol (1.47 fold), (-)-epicatechin (1.533 fold), and epigallocatechin (2.031 fold). Only O-acetyl salicylic acid showed 2.916-fold decrease in response to shade treatment on tea leaves. Other important metabolites also showed increasing trend in responses such as D-glucose-6-phosphate (0.65-fold), L-valine (1.933-fold), L-glutamic acid (2.597-fold), 2-isopropylmalic acid (0.654-fold), L-glutamic acid (dehydrated) (3.035-fold), DL-3,4-dihydroxyphenylglycol (0.570-fold), L-proline (12.01-fold), D-glucic acid (0.59-fold), D-saccharic acid (11.907-fold), and norvaline (12.241-fold). Only uracil (1.156-fold), mucic acid (1.457-fold) and galactolin (14.962-fold) showed decreasing tendency in concentration.

Tea plants are adapted to shade conditions under natural habitat, so sensitivity to strong light is predictable. Chemical composition analyses in this study indicated notable increase in phenols, flavonoids, amino acids, and some sugars under shade condition of tea bushes than plants grown under unshade condition, that is, under sunlight-exposed condition. The phenolic compounds, namely, gallic acid, quinic acid, pyrogallol, chlorogenic acid, catechin, epicatechin, and epigallocatechin are derived from the shikimic acid pathway, located upstream of the phenylpropanoid pathway in plants (Hermann, 1995). Whereas when the plants are exposed to direct sunlight, UV radiation cause cell destruction and the UV stress may result in the synthesis of cell-protective compounds (flavonoids, phenol, amino acids) in tea leaves but in lesser concentration than the shade-treated plants.

When we compared between thiometoxam treated shade and unshade plants (Figure 9), interestingly it was observed that most of the phenolic compounds, many organic acids, some sugars and sugar alcohols and fatty acids showed decrease in concentration from shade treatment than unshade treatment. Phenolics such as pholoroglucinol, DL-3,4-dihydroxyphenylglycol, alizarin, 4-isopropyl benzoic acid, gallic acid, shikimic acid, piceatannol, pyrogallol and catechin showed 11.234-, 3.4-, 2.57-, 1.995-, 1.77-, 1.585-, 1.319-, 1.132- and 1.037-fold decreases, respectively due to chemical pesticide treatment in shade plants than chemically treated unshade plants. Although some phenolics like O-acetylsalicylic acid, epicatechin, epigallocatechin, (-)—epicatechin showed increasing pattern in their concentration in a manner of 12.325-, 3.005-, 2.706- and 2.170-fold increase, respectively. Sugars and sugar alcohols such as galactolin, D-mannitol, D-mannose, D-lyxose, and fructose showed 14.636-, 1.650-, 1.880- and 1.119-fold decrease in concentration. Oleic acid showed 13.525-fold and uracil showed 2-fold decrease due to chemical pesticide and shade treatment together. Organic acids like D-saccharic acid (~10.953-fold), citric acid (~18.1-fold), lactobionic acid (~1.791-fold), glyceric acid (~1.48-fold), fumaric acid (~1.26-fold) and tartaric acid (~13.45-fold) also showed a decreasing trend in concentration.

The amino acids can function as the precursors of secondary metabolites that can protect plants from various diseases (Fiehn, 2006). In this study, due to chemical and shade treatment of tea plant, the leaf metabolites showed increase in metabolite level among all the amino acids. Norvaline (10.829-fold), L-proline (19.578-fold), L-valine (11.109-fold), L-alanine (2.303-fold), L-allothreonine (1.863-fold), L-mimosine (1.597-fold), L-glutamic acid (1.362-fold) and L-glutamic acid (dehydrated) (1.452-fold) showed maximum fold increase in metabolite concentration.
The luminous intensity of shade and unshade conditions could not be measured as light reaching different parts of tea bushes are different and it also depends on the canopy formed by different shade trees at different seasons and the shade trees are deciduous in most of the cases. But the knowledge of shading effects on tea shoots is important for the improvement of tea quality and productivity. Leaf thickness, leaf mass area, leaf density of new leaves were smaller under shade culture than under open or unshade culture. Also chlorophyll, epicatechin and epigallocatechin are strongly related with shading effects (Sano et al., 2018). From the above study, it can be said that the shading cultivation may be beneficial for high quality and high productivity in tea bushes in tea gardens.

A change in DPPH radical scavenging activity among the unshade control versus unshade pesticide treated samples and shade control versus shade pesticide treated were found significantly (p-value < 0.05) different by t-test (p-values showing 0.000259 and 0.000235) and also in case of superoxide radical scavenging activity (p-values showing 0.0197 and 0.002) among types of samples of plant extracts were noticeable. No statistically significant change was observed in their total antioxidant capacity.

7. Conclusion
This study provides an insight into the change in metabolite network of first flush leaf samples of Camellia sinensis cv. TV 26 for the first time, whose plants have been exposed to shade, unshade, shade pesticide and unshade pesticide treatments. Using GC/MS based metabolomic and chemometric approaches the changes in metabolite level due to different treatments on tea plants have shown that there is increase in phenols, amino acids, organic acids, sugar alcohol concentrations when plants are exposed to shade treatment than unshade treatment. On the other hand during pesticide treatment on shade tea plants, there is decrease in many important metabolites especially phenolics and organic acids, which impart the major flavor, taste, aroma and quality of the beverage, but it is interesting enough to note the increase in amino acids, which cope the plants to protect them from chemical pesticide treatments under shade conditions.

There is very limited information on antioxidant capacities of shade, unshade, shade pesticide treated, and unshade pesticide treated first flush tea leaf samples. Therefore, from this study, it can be concluded that shade treatment may be beneficial for the tea cultivation than unshade condition as many important metabolites are increasing due to shade conditions and in some cases antioxidant activities are also found higher in shade treated plants samples than unshade condition. However, more studies are required to be done. In this study, we have highlighted not only the changes in metabolites but their antioxidant capacities using multiple treatment procedures on first flush tea leaf samples.

Abbreviations
GC/MS: Gas Chromatography Mass Spectrometry; UV: Ultra Violet; MOX: methoxyamine hydrochloride; MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide; MO: methyl octanoate; MD: methyl decanoate; ML: methyl laurate; MAME: myristic acid methyl ester; MP: methyl palmitate; SAME: stearic acid methyl ester; MA: methyl arachidate; MB: methyl behenate; MT: methyl tetracosenoate; MH: methyl hexacosanoate; MSD: Mass detector; FAME: fatty acid methyl ester; IRI: internal retention index; RT: Retention Times; RI: Retention Indices; AMDIS: Automated mass spectral deconvolution and identification system; RTL: retention time locking; DPPH: 1,1-diphenyl-2-picrylhydrazyl; EDTA: Ethylene diamine tetra acetic acid; NBT: Nitro blue tetrazolium; TAC: Total antioxidant capacity; MVA: Multivariate data analysis; RRR: Relative response ratio; PCA: Principal Component Analysis; PLS-DA: Partial Least Square Discriminant Analysis; s-PLS-DA: sparse Partial Least Square Analysis; OPLS-DA: Orthogonal Partial Least Squares Discriminant Analysis; VIP: variable importance in projection; ANOVA: one-way analysis of variance; HSD: Honestly significant difference test; MUFAs: Mono unsaturated fatty acid; PUFA: Poly unsaturated fatty acid.

Authors’ contribution
SR and SD together conceived and designed the research project; SR and AG conducted the treatment of tea leaves with pesticide, shade and unshade conditions, collected and prepared the samples and supervised the whole experiment. JC and SD prepared and analyzed the GC/MS-based research. SD analyzed the data and interpreted results chemometrically and wrote the manuscript. All authors read and approved on the final version of the manuscript.

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References
Allwood, J. W., Ellis, D. I., & Goodacre, R. (2008). Metabolomic technologies and their application to the study of plants and plant-host interactions. Physiologia Plantarum, 132(2), 117–135.
Balakrishnan, V., Ravindran, K. C., Venkatesan, K., & Karuppusamy, S. (2005). Effect of UV-B supplemental radiation on growth and biochemical characteristics in Crotalaria juncea L. seedling. Electronic Journal of Environmental, Agricultural and Food Chemistry, 4(6), 1125–1131. http://ejefach.euvigo.es/ejefach/2005/006462005.pdf
Bonejyee, B. (1979). Intra-tree variation in the distribution of the tea red spider mite Oligonychus coffeae (Nietner). Acarologia, 21(2), 216–220.
Borua, D. N. (2007). Science and Practice in Tea Culture (1 ed.). Tea Research Association.
Beauchamp, C., & Fridovich, I. (1977). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry, 44(1), 276–287. https://doi.org/10.1016/0003-2697(77)90370-8
Beddage, H. P., & Mohotti, A. J. (2005). A compilation of potential alternate shade tree species for tea plantations. Proceedings of the tenth annual forestry & environmental science symposium, department of forestry & environmental science, Plant Physiology Division, Tea Research Institute of Sri Lanka, Talawakele. University of Sri Jayewardenepura, Sri Lanka24.
Bhattacharya, S., & Sen-Mandi, S. (2011). Variation in antioxidant and aroma compounds at different altitude: A study on tea (Camellia sinensis L. Kuntze) clones of Darjeeling and Assam, India. African Journal of Biochemistry Research, 5(5), 148–159. https://doi.org/10.5897/AJBR.9000260
Braco, A., Nunziatina De, T., Lorenzo Di, B., Cosimo, P., Mateo, P., & Ivano, M. (2001). Antioxidant principles from Bauhinia terapotensis. Journal of Natural Product, 64(7), 892–895. https://doi.org/10.1021/np010084s
Chen, H., Pan, M., Liu, X., & Lu, C. (2017). Evaluation of transfer rates of multiple pesticides from green tea into infusion using water as pressurized liquid extraction solvent and ultra-performance liquid chromatography tandem mass spectrometry. Food Chemistry, 216, 1–9. https://doi.org/10.1016/j.foodchem.2016.07.175
Das, G. M. (1959a). Bionomics of the tea red spider, Oligonychus coffeae (Nietner). Bulletin of Entomological Research, 50(2), 265–274. https://doi.org/10.1017/S0007485300054572
Das, S., Dutto, M., Chaudhury, K., & De, B. (2016). Metabonomics and chemometric study of Acras sapata L. fruit extracts for identification of metabolites contributing to the inhibition of α-amylase and α-glucosidase. European Food Research Technology, 242(5), 733–743. https://doi.org/10.1007/s00217-015-2581-0
Dasgupta, N., & De, B. (2004). Antioxidant activity of piper betle L. leaf extract in vitro. Food Chemistry, 88(2), 219–224. https://doi.org/10.1016/j.foodchem.2004.01.036
Dunn, W. B. (2008). Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. Physical Biology, 5(1), 1–24. doi:10.1088/1478-3975/5/1/011001
Fiehn, O. (2006). Metabolite profiling in Arabidopsis. In J. Salinas & J. J. Sanchez-Serrano (Eds.), Methods in molecular biology. 323 (pp. 439–447) Arabidopsis protocols (2nd ed.). Humana Press Inc.
Fiehn, O., Kopka, J., Darmann, P., Altmann, T., Trethewey, R. N., & Willmitzer, L. (2000). Metabolite profiling for plant functional genomics. Nature Biotechnology, 18(11), 1157–1161. https://doi.org/10.1038/sj.nbt.1501858
Herrmann, K. M. (1995). The shikimate pathway as an entry to aromatic secondary metabolism. Plant Physiology, 107(1), 7–12. https://doi.org/10.1104/pp.107.1.7
Katsuno, T., Kasuga, H., Kusano, Y., Yaguchi, Y., Tomomura, M., Cui, J., Yang, Z., Baldermann, S., Nakamura, Y., Ohnishii, T., Mase, N., & Watanabe, N. (2014). Characterisation of odorant compounds and their biosynthesis in green tea (Camellia sinensis) leaves by single light wavelength. Scientific Reporter, 5(1), 16858. https://doi.org/10.1038/srep16858
Kessler, A., & Baldwin, I. T. (2002). Plant responses to insect herbivory: The emerging molecular analysis. Annual Review of Plant Biology, 53(3), 299–328. https://doi.org/10.1146/annurev.arplant.53.100301.135207
Kind, T., Wohlgeuth, M., Du Lee, Y., Lu, Y., Palazoglu, M., Shahbaz, S., & Fiehn, O. (2009). Fiehnlib-mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. Analytical Chemistry, 81(24), 10038–10048. https://doi.org/10.1021/ac9019522
Krishnan, P. (2005). Metabolite fingerprinting and profiling in plants using NMR. Journal of Experimental Botany, 56(4), 255–265. https://doi.org/10.1093/jxb/eri010
Lesser, M. P. (1996). Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. Limnology and Oceanography, 41(2), 271–283. https://doi.org/10.4319/lo.1996.41.2.0271
Mruthunjaya, D., Joshi, H., & Nalini, M. S. (2016). Phytochemicals, antioxidative and in vivo hepatoprotective potentials of litsea floribunda (BL.) Gamble (Lauraceae) - An endemic tree species of the Southern Western Ghats, India. Jordan Journal of Biological Sciences, 9(3), 163–171.

Paré, P. W., & Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. Plant Physiology, 121(2), 325–331. https://doi.org/10.1104/pp.121.2.325

Priede, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. Analytical Biochemistry, 269(2), 337–341. https://doi.org/10.1006/abio.1999.4019

Rohloff, J. (2015). Analysis of phenolic and cyclic compounds in plants using derivatisation techniques in combination with GC/MS based metabolite profiling. Molecules, 20(2), 3431–3462. https://doi.org/10.3390/molecules20023431

Sano, T., Horie, H., Matsunaga, A., & Horono, Y. (2018). Effect of shading intensity on morphological and color traits, and chemical components of new tea (Cameilla sinensis L.) shoots under direct covering cultivation: Changes in morphology, color, and chemical components of tea shoots due to shade. Journal of the Science of Food and Agriculture, 98(15), 5666–5676. https://doi.org/10.1002/jsfa.9112

Spratlin, J. L., Serkova, N. J., & Eckhardt, S. G. (2009). Clinical applications of metabolomics in oncology: A review. Clinical Cancer Research, 15(2), 431–440. https://doi.org/10.1158/1078-0432.CCR-08-1059

Trehewey, R. N., Krotzky, A. J., & Willmitzer, L. (1999). Metabolic profiling: A Rosetta stone for genomics? Current Opinion in Plant Biology, 2(2), 83–85. https://doi.org/10.1016/S1369-5266(99)80017-X

Vinayavekhin, N., Homan, E. A., & Saghatelyan, A. (2010). Exploring disease through metabolomics. ACS Chemical Biology, 5(1), 91–103. doi: 10.1021/ cb900271r

Yang, Z., Kobayashi, E., Katsuno, T., Asanuma, T., Fujimori, T., Ishikawa, T., Tomomura, M., Machizuka, K., Watase, T., Nakamura, Y., & Watanabe, N. (2012). Characterisation of volatile and non-volatile metabolites in etiolated leaves of tea (Camellia sinensis) plants in the dark. Food Chemistry, 135(4), 2268–2276. https://doi.org/10.1016/j.foodchem.2012.07.066

Ye, G. Y., Xiao, Q., Chen, M., Chen, X. X., Yuan, Z. J., Stanley, D. W., & Hu, C. (2014). Tea: Biological control of insect and mite pests in China. Biological Control, 68(1), 73–91. http://dx.doi.org/10.1016/j.biocontrol.2013.06.013

Zeng, L., Zhou, Y., Gui, J., Fu, X., Mei, X., Zhen, Y., Ye, T., Du, B., Dong, F., Watanabe, N., & Yang, Z. (2016). Formation of volatile tea constituent indole during the oolong tea manufacturing process. Journal of Agricultural and Food Chemistry, 64(24), 5011–5019. https://doi.org/10.1021/acs.jafc.6b01742

Zhou, Y., Zeng, L., Liu, X., Gui, J., Mei, X., Fu, X., Dong, F., Tang, J., Zhang, L., & Yang, Z. (2017). Formation of (E)-nerolidol in tea (Camellia sinensis) leaves exposed to multiple stresses during tea manufacturing. Food Chemistry, 231, 78–86. https://doi.org/10.1016/j.foodchem.2017.03.122
