

Leading progress on genomics, health benefits and utilization of tea resources in China



AUTHORS

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China is the largest producer and consumer of tea in the world and has abundant tea plant resources and tea products (including green tea, black tea, dark tea, oolong tea, white tea and yellow tea). Since the beginning of the new century, scientific research and technological innovation have led to improvements in the output, quality, safety and consumption of tea in China, which has driven the healthy and sustainable development of China's tea industry. The team of Academician professor Zongmao Chen at the Tea Research Institute of Chinese Academy of Agricultural Sciences, the team of professor Zhonghua Liu at Hunan Agricultural University (HAU) and the team of professor Lizhi Gao at South China Agricultural University (SCAU) have made a series of outstanding achievements in research fields such as tea plant genomics and resource utilization, the theory of tea processing, tea and health, the utilization of functional components from tea, tea pesticide residues and safety.

TEA PLANT GERMLASM AND GENOMICS

The tea plant *Camellia sinensis* (L.) O. Kuntze is the source of commercially grown tea, belongs to the genus *Camellia* (Theaceae), and has numerous secondary metabolites that give rich flavours and health benefits. However, the genetic bases for the biosynthesis and rich accumulation of all forms of the bioactive compounds in tea, which affect the quality and production of tea products, have largely relied on tea plant genomics. Lizhi Gao leads a team that first examined the genome size, variation and evolution among *Camellia* representatives of almost all sections of the two subgenera, *Camellia* and *Thea*¹. Gao's group launched the first tea plant genome project in 2010 based on an accurate estimation of the DNA content of *C. sinensis* var. *assamica* to be $1C = 3.01$ pg, which is equal to a genome size of about 2,940 Mb. After seven years of work to overcome the challenge of a high level of genomic heterozygosity and large genome size, the team completed the 3.02-gigabase genome assembly of Yunkang 10

($2n = 2x = 30$ chromosomes), a diploid elite cultivar of *C. sinensis* var. *assamica* widely grown in southwestern China based on sequence data from whole-genome shotgun sequencing². Repetitive sequences account for ~80.89% of this large diploid genome, while the rest yields 36,951 high-confidence protein-coding genes. Long terminal repeats (LTR) retrotransposons represent the majority (~67.21%) of the tea plant genome, of which about 55.09% of the assembly was identified as Ty1/*cop* and Ty3/*gypsy* superfamilies. Ty3/*gypsy* LTR retrotransposon families (~47.08%) predominate and contribute most to the tea plant genome expansion. The largest Ty3/*gypsy* retrotransposon family, TL001, accounts for ~36.79% of the whole tea plant genome, representing the first report of a single LTR retrotransposon family propagating and persisting for more than 50 million years of evolution. They identified lineage-specific expansion of gene families associated with flavonoid metabolic biosynthesis that enhance the production

of catechins, the activation of terpene enzymes associated with tea flavour, and disease defence and environmental stress tolerance genes facilitating adaptation to diverse habitats. Comparative genomic analysis of N-methyltransferases demonstrates an independent, recent and rapid evolution of the caffeine synthetic pathway in the tea plant relative to cacao and coffee trees. Recently, Gao's group generated the first high-quality reference genome for the commercial *Biyun* variety of *C. sinensis* var. *sinensis* using a single-molecule real-time sequencing (SMRT) platform. The final assembly of the tea plant genome was 2.92 Gb, accounting for ~96.69% of the estimated genome size of 3.02 Gb. The assembly comprises 9,998 scaffolds with scaffold N50 and contig N50 lengths of 1.16 Mb and 707.7 Kb, respectively, ~2.58 and 35.46 times longer than the previously reported genome assembly of *Yunkang 10* (449.457 Kb and 19.96 Kb) that was assembled by Illumina reads only¹. At least 74.13% of the 3.02-Gb genome assembly consists of

repetitive sequences and the rest produces 40,163 high-confidence predictions of encoded proteins. Gao's group obtained the fourteen *Camellia* cp genomes, including both *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*, providing the first insights into the cp genome variation and evolution in this genus³. Gao's group also assembled the complete mt genomes of *C. sinensis* var. *assamica* cv. *Yunkang 10* in two circular contigs of 702,253 bp and 178,082 bp in length, respectively, which comprise of 35 protein-coding genes, 29 tRNAs, and two rRNAs. Great progress in tea plant genomics will facilitate marker-assisted selection of tea plant improvement and utilization of alien genes derived from the abundant diversity of tea plant germplasm that would offer promise to develop better tea cultivars of high quality.

Despite the acquired knowledge that differential accumulation of the three characteristic secondary metabolites – catechins, theanine and caffeine – in tea leaves that largely determine tea qualities, more efforts are needed regarding the complex transcriptional regulation of these metabolic pathways. Gao's group performed genomic, transcriptomic and metabolomic analyses towards determining the genetic basis of tea-processing suitability, flavours and health benefits that make tea one of the world's most highly consumed beverages². The genus *Camellia*, consisting of ~119 species with differential metabolite profiles, offers a uniquely powerful system for dissecting the variation and evolution of these pathways that determine tea-processing suitability. Comparisons among representative *Camellia* lineages show that, despite the conservation of the three

metabolic pathways, significantly higher expression levels of flavonoid- and caffeine- but not theanine-related genes are responsible for the increased production of catechins and caffeine in the cultivated tea plant and close wild relatives. These findings establish fundamental knowledge regarding the genomic basis of tea-processing suitability, tea palatability and health benefits.

THE ALBINO MECHANISM OF 'ANJI BAICHA' TEA

The germplasm resource of the tea plant is abundant in China, and a large number of mutants provide rich genetic resources for finding and utilization of novel genes in *C. sinensis*. The 'Anji Baicha', an albino mutant tea cultivar, has a reversible albino phenotype at low temperatures⁴. Albino 'Anji Baicha' contains high levels of amino acids. The content of L-theanine in 'Anji Baicha' is two times that found in normal cultivars when it is in the albinistic stage. Liu and Huang's group determined the mechanism of albinism and amino acid enrichment in 'Anji Baicha' based on different research levels. The subcellular studies revealed that the transition of etioplast-chloroplast was blocked and the grana in the chloroplast in the newly developed leaves of 'Anji Baicha' were destroyed when the environment temperature was lower than 19°C, leading to abnormal development of the chloroplast in 'Anji Baicha'. However, the ultra-structure of chloroplast in 'Anji Baicha' returned to normal when the environment temperature was higher than 19°C⁴. Suppression subtractive hybridization (SSH), cDNA-amplified fragment length polymorphism (cDNA-AFLP) and RNA sequencing (RNA-Seq) were performed to identify the genes that were differentially expressed

during periodic albinism in 'Anji Baicha'. The results showed that the transcripts, containing transcription factors, ubiquitination-related genes, chloroplast biogenesis genes, signal transduction genes, stress-related genes, cell cycle genes, and carbohydrate and energy metabolism genes were differentially expressed in four patterns. The researchers found up-regulation in the albinistic stage and down-regulation in the albinistic stage. They also found that expression levels slowly rose in the pre-albinistic stage, the albinistic stage and the regreening stage, and that expression levels slowly drop in the pre-albinistic stage, the albinistic stage and the regreening stage⁵. The proteomic researches indicated that the protein spots, involved in the metabolism of carbohydrate and energy metabolism, photosynthesis, protein processing, stress defense and RNA processing, were differentially expressed in leaves of 'Anji Baicha' in the pre-albinistic stage, the albinistic stage and the regreening stage⁴. Furthermore, it was reported that the activity of phenylalanine ammonia-lyase (PAL), Cinnamate 4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL) were co-regulated in 'Anji Baicha' during the periodic albinism⁶.

ANTI-SENESCENCE PROPERTIES OF TEA

Tea has been used in drinks for more than 3,000 years in China. Tea consumption has also been associated with health benefits including for delaying senescence and extending lifespan in folklore. Liu's group has focused on tea and its active ingredients to observe if it is possible to influence the lifespan of the

nematode *Caenorhabditis elegans*, a model organism for aging, to provide insight into human senescence and age-related diseases⁷. Liu's group found that Epigallocatechin-3-gallate (EGCG), the most abundant and widely studied catechin in tea, prolonged a healthy lifespan mostly during early to mid-adulthood, but not at older ages nor at the development stage. The effect of EGCG on the lifespan of *C. elegans* reflected a nonlinear or inverted U-shaped dose-response relationship. According to the free-radical theory of senescence, EGCG has been widely studied for potentially beneficial effects as an antioxidant. EGCG induced a transient increase in reactive oxygen species (ROS), and the lifespan-extending ability was abolished by NAC and BHA, two different principles of antioxidants. AAK-2, the AMPK ortholog, required for the induction of ROS and mitochondrial biogenesis, was essential for EGCG-induced longevity. To verify this finding, we then assessed the effect of EGCG on growth of *Escherichia coli* (OP50). The hormetic response to the bactericidal effect of EGCG was observed in LB broth at 0.4-fold dilution. The induced intracellular ROS, in contrast to extracellular ROS, mediated EGCG-induced OP50 inhibition at high dose. Altering or killing *E. coli* also affected the lifespan of *C. elegans* induced by EGCG⁸. The other focus of Liu's group is the capacity of tea and its bioactive substances to resist environmental stress in living organisms. The group found that black tea extracts did not increase lifespan under normal conditions. Black tea extracts protected *C. elegans* against osmosis, heat shock and ultraviolet irradiation.

Black tea was more effective than green tea in increasing the survival of *C. elegans* under hypertonic conditions⁹.

Maintaining the protein homeostasis of cells in a timely and effective manner is key to achieving healthy senescence. Liu's group found that functional compounds in tea can effectively inhibit the formation of aggregates, down-regulate the expression of senescence genes and delay cell senescence. *In vitro* experiments have shown that both catechin monomers (EC, EGC, ECG, EGCG) and theaflavin monomers (TF, TF-3-G, TF-3'-G, TFDG) can inhibit the formation of senile pigments, glycosylated proteins, and β -amyloid proteins by retarding the formation of the amyloidogenic β -sheet-rich structures. The conjugative effect and galloyl group of tea polyphenols are closely related to the activity of inhibiting aggregate formation¹⁰. *In vivo* experiments in mice showed that through interfering with the formation of p62 and ubiquitin-conjugated protein aggregates, EGCG and EGCG3'Me can maintain the cell protein degradation system, intervene in the RAGE/NF- κ B pathway of chronic inflammation, down-regulate the expression of the senescence gene p16^{INK4}, showing strong activities in retarding the progression of brain aging and diabetic nephropathy¹¹. 3,3'-azanediybis (4-hydroxybenzoic acid) (CDT-1) is a microbial metabolite of protocatechuic acid, and 8-C N-ethyl-2-pyrrolidinone substituted flavan-3-ols (CDT 2) is one of the microbial conversion products of (+) catechin, (-) epicatechin and theanine in Chinese dark tea. Senescence-accelerated mouse prone 8 (SAMP8) mice were treated with CDT-1, CDT-2 and L-theanine for

14 weeks by gavage. The result revealed that CDT-1, CDT-2 and L-theanine can inhibit weight loss due to aging, increase endogenous antioxidant capacity, block the formation cycle of the oxidative stress-amyloidogenic protein, down-regulate the expression of senescence genes such as PS1, p16^{INK4} and p21, and inhibit the apoptosis of neuronal cells. The data show that the protective activity of delaying ageing of SAMP8 neurons was excellent and the activity of CDT-2 was better than that of CDT-1 and L-theanine¹². These findings reveal that maintaining protein homeostasis may be a potential mechanism for tea to delay the development of degenerative changes caused by ageing or stress-induced premature senescence.

HYPOLIPEMIC AND ANTI-OBESITY EFFECTS OF DARK TEA

Dark tea is a special type of Chinese tea in which microbial fermentation takes place during processing. Fuzhuan brick tea, the most popular dark tea, has been consumed daily in northwest China for several hundred years. *Eurotium cristatum* is the dominant fungus used for post-fermentation. Research results of Liu's group show that Fuzhuan brick tea could regulate lipid metabolism, improve intestinal function and prevent metabolic syndromes such as obesity and hyperlipidemia from the levels of biochemical metabolism, cytology, gene expression, proteomics and intestinal microecology.

Based on the models of obese and hyperlipidemia rats, the hypolipidemic and anti-obesity effects of Fuzhuan brick tea water extract was studied. The results showed that Fuzhuan brick

tea water extract significantly suppressed the increase of body weight, accumulation of adipose tissue, and elevated the level of blood lipids in obese rats fed a high-fat diet. Moreover, Fuzhuan brick tea water extract attenuated the gene expressions of fatty acid synthase, sterol regulatory element binding protein-1c (SREBP-1c) and CCAAT/enhancer binding protein α (C/EBP- α), which is related to lipogenic metabolism and adipose differentiation. In contrast, the gene expressions of hepatic peroxisome proliferator-activated receptor α (PPAR α), carnitine palmitoyltransferase 1a (CPT1a) and LDL receptor (LDLR) involved in energy expenditure and lipolysis were increased by Fuzhuan brick tea water extract treatment¹³. Quantitative proteomics analysis also showed that Fuzhuan brick tea water extract significantly down-regulated the protein expressions of acetyl-CoA carboxylase 2 (ACACB), fatty acid synthase and glycerol-3-phosphate dehydrogenase (GPD1), which is related to fatty acid synthesis and lipogenesis. Also, the protein expressions of CPT1a, enoyl-CoA hydratase (ECHS1) and hydroxyacyl-CoA dehydrogenase (HADH) involved in FA β -oxidation were up-regulated by treatment with Fuzhuan brick tea water extract. Thus, lipid metabolism was regulated by the inhibition of fatty acid and fat synthesis and the enhancement of FA β -oxidation and energy consumption. Some putative drug targets were also revealed that could attenuate deleterious effects of metabolic diseases on liver such as ACACB, fatty acid synthase and ECHS1¹⁴. Additionally, the results of studies in which volunteers drank Fuzhuan brick tea every

day also support the conclusion of animal studies that Fuzhuan brick tea can regulate blood lipid levels¹⁵. The water extract of *E. cristatum* in Fuzhuan brick tea can significantly inhibit fat deposition in 3T3-L1 adipocyte and *C. elegans*.

Gut microbiota play an important part in maintaining intestinal function and regulating lipid metabolism. The polysaccharides from Fuzhuan brick tea (FBTPS) were firstly separated and purified, and then *in vitro* digestion of FBTPS under simulated saliva, gastric and small intestinal conditions were investigated and mice fed a high-fat diet were used to evaluate the effect of FBTPS on metabolic syndrome and gut microbiota *in vivo*. The results showed that FBTPS could pass through the digestive system without being broken down and could reach the large intestine safely, where the polysaccharides could be broken down and utilized by gut microbiota. Also, FBTPS intervention could significantly attenuate metabolic syndrome in mice fed a high fat diet, restore the phylogenetic diversity of gut microbiota, and reverse the high-fat-diet-induced increases in relative abundances of *Erysipelotrichaceae*, *Coriobacteriaceae* and *Streptococcaceae* and the significant changes in relative abundances of 44 key operational taxonomic units negatively or positively associated with metabolic syndrome. Thus, the indigestible FBTPS reach the large intestine and then modulate the gut microbiota and lipid metabolism, which might be one of the possible mechanisms by which Fuzhuan brick tea¹⁶ helps to prevent metabolic syndrome.

THE QUALITY FORMATION OF DARK TEA DURING PROCESSING

Microbial fermentation is important for providing the flavour and quality of dark tea. The manufacturing process of dark tea is divided into primary processing and press processing. The procedure of primary processing is as follows: fresh tea leaves → fixing → rolling → piling fermentation → drying → primary dark tea. The procedure of press processing is as follows: primary dark tea → screening → blending → steaming → piling fermentation → pressing → fungal fermentation → drying → brick dark tea. Fuzhuan brick tea is a particularly special type of dark tea because *E. cristatum* is the dominant microbe during processing. Piling fermentation and fungal fermentation are the key steps in the manufacturing process of primary dark tea and Fuzhuan brick tea, respectively. The variations in microbial community, major chemical compounds and the mechanism of characteristic quality formation of primary dark tea and Fuzhuan brick tea were clarified by Liu's group.

For the pile fermentation of primary dark tea, the genera *Cyberlindnera*, *Aspergillus*, *Uwebraunia*, and unclassified *Pleosporales* of fungus and *Klebsiella*, *Lactobacillus* of bacteria were predominant in the early stage of the process, but only *Cyberlindnera* and *Klebsiella* dominated in the late stage and maintained relatively constant until the end of fermentation. In addition, the fungi made more contributions to the formation of characteristic flavour of primary dark tea than bacteria during the pile fermentation. Furthermore, ten microbial genera – *Cyberlindnera*, *Aspergillus*, *Eurotium*, *Uwebraunia*,

Debaryomyces, *Lophiostoma*, *Peltaster*, *Klebsiella*, *Aurantimonas* and *Methylobacterium* – were identified as core functional genera for the pile fermentation of primary dark tea¹⁷. The activities of polyphenol oxidase, cellulase and pectinase were increased, while the major chemical compounds, including tea polyphenols, catechins, amino acids, flavonoids, soluble sugars were decreased during this process. Amino acid was identified as the important factor in shaping the microbial community structure of the primary dark tea ecosystem¹⁷.

For the manufacturing process of Fuzhuan brick tea, the fungal genera *Aspergillus*, *Cyberlindnera* and *Candida* were predominant at the early stage of the manufacturing process, but after fungal fermentation for three days only *Aspergillus* was dominant. The fungal community made more contribution than the bacterial community to the characteristic quality formation of Fuzhuan brick tea¹⁸. The dominant fungal species was identified as *E. cristatum* (*Aspergillus cristatum*) by the multilocus sequence typing method. The bacterial genera *Klebsiella* dominated at the beginning of the process stage, but was quickly replaced by *Pseudomonas*, *Lactococcus*, *Stenotrophomonas*, *Enterococcus* and *Bacillus* at the later stage and remained stable until the end of the process. The activities of polyphenol oxidase, cellulase and pectinase were increased during the fungal fermentation process, the activity of polyphenol oxidase and cellulase activity reached a peak at nine days, and the activity of pectinase reached the peak at 12 days. The contents of tea polyphenols, catechins, amino acids, flavonoids, organic acids and soluble sugars were significantly decreased,

while gallic acid content was significantly increased during the manufacturing process. GCG, EGCG and GA (gallic acid) as well as the interactions among them were the ones that probably influenced, or were influenced by, the fungal communities during the fermentation process of Fuzhuan brick tea¹⁸. Therefore, the essence of pile fermentation of primary dark tea and fungal fermentation of Fuzhuan brick tea is based on the metabolism of microorganisms. The chemical component of tea leaves was transformed by the microbial extracellular enzymes of tea leaves, which shape the special flavour of dark tea and Fuzhuan brick tea during the manufacturing process. The dominant fungal species *E. cristatum* was a key factor in forming the fungal aroma in Fuzhuan brick tea.

EFFICIENT UTILIZATION OF FUNCTIONAL COMPONENTS FROM TEA

Research into the functional ingredients of tea started about 30 years ago in China. Technological innovation on the extraction, separation and purification of active components from tea, especially polyphenols, has promoted the global development of tea comprehensive processing (the extraction of active ingredients, such as instant tea, tea polyphenols, caffeine, theanine and theaflavins from tea leaves to get the soluble extracts of tea leaves) and tea extracts applied in the field of health. Liu's team has systematically studied new technologies for the extraction, separation and purification of tea components over the past 20 years. Liu's team has effectively improved the technical level

and international force of the Chinese tea comprehensive processing industry.

Extraction, separation, and purification of tea polyphenols.

Column chromatography technology with macroporous adsorption resin, and nanomembrane technology were applied into the industrial production of tea catechins by Liu's team to isolate and purify catechins and remove caffeine efficiently with water and edible alcohol as solvents only. The potential risks of harmful solvent residues such as ethyl acetate, trichloromethane or dichloromethane in traditional manufacture processes was effectively eliminated.

Innovative separation and purification technology of tea catechins monomers.

By comprehensively adopting enzyme engineering and column chromatography and online monitoring technologies, the technical bottleneck of high-efficiency industrial separation and preparation of tea catechin monomers was overcome by Liu's team. The annual production capacity of EGCG 90%-98% in China is in excess of 30 tons.

Industrial production of theaflavins by enzymatic oxidation of tea catechins.

Liu's team screened polyphenol oxidase from different plants (such as tea, pear and apple) and microbial sources, constructed a new system for the industrial production of theaflavins by enzymatic oxidation of green tea catechins, and established the large-scale separation and purification technology system of high purity theaflavin and its monomers. This breakthrough has overcome the issue of production cost of theaflavins from black tea.

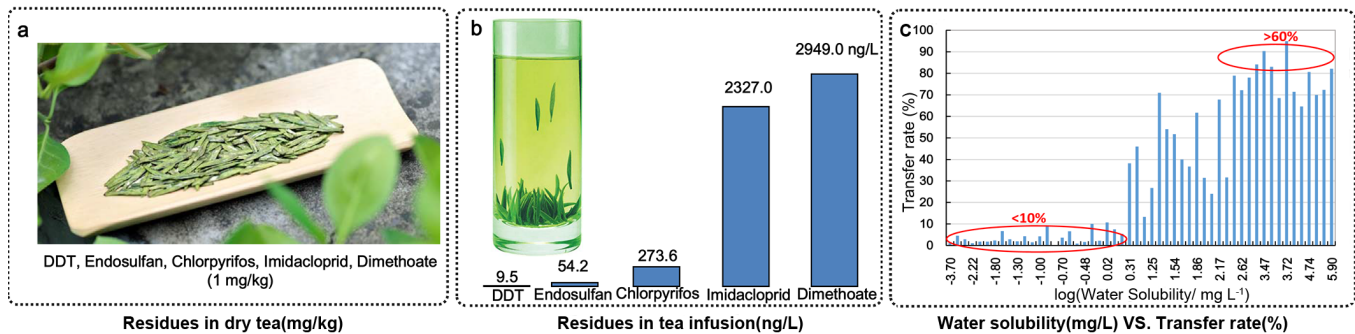


Figure 1: Transfer of pesticide residues from dry tea to tea infusion.

Separation and purification of natural L-theanine. Liu's team researched the separation and purification of natural L-theanine from the water layer of tea polyphenols extracted by ethyl acetate, and implemented the manufacture of 20%-98% natural L-theanine on a large scale for the international market.

CRITICAL CONSIDERATION ON THE IMPORTANT FACTOR INFLUENCING CONSUMER SAFETY IN TEA

Tea is a drink that is believed to confer health benefits. However, because chemical pesticides are needed to protect tea plants from harmful organisms, pesticide residues in tea are considered to be a health concern. Safety is a universal principle in tea garden management in tea-producing countries. Maximum pesticide residue limits are taken seriously by professional tea growers. Research by Chen's group showed that the methodology used to determine the maximum residue limits of pesticides in tea is the same as the methodology applied to determining maximum pesticide residues in foods such as rice, vegetables, fruit and meats. However, in the tea drinking process, dry tea is brewed with boiling water and then the tea infusion is swallowed but the infused tea leaves are

discarded. Chen's group asked whether the residue level of pesticides in the tea brew was equal to that in dry tea. They found residue differences in tea infusion (9.5-2949.0 ng/L, **Figure 1b**) after the dry tea incurred by pesticides of 1 mg/kg (**Figure 1a**) was brewed. The effect of water solubility on transfer rate of pesticides from dry tea to tea infusion (**Figure 1c**) suggested that only a portion of residual pesticides in dry tea would transfer to the tea infusion^{19,20}. Therefore it is possible that there could be an overestimation of the intake of pesticide residues from dry tea, especially for pesticides with low water solubility.

The topic of 'maximum residue level fixation based on the residue in tea infusion' was discussed and accepted by delegates from the tea-producing and tea-importing countries at the Food and Agriculture Organisation Intergovernmental Group on Tea in 2012. In 2016, it was accepted as a conclusion in the 48th Food and Agriculture Organisation/World Health Organization Codex Committee on Pesticide Residues meeting report as follows: The FAO representative of the joint meeting on pesticide residues (JMPR) informed the committee that the use of available tea brew

studies to estimate processing factors was part of the current JMPR procedures for the establishment of maximum residue levels for dry tea leaves and encouraged countries when submitting their trials to also include tea brew studies. By using this idea, six maximum residue levels in tea, including endosulfan in CAC and EPA, cypermethrin in CAC, bifenthrin in EPA, indoxacarb in CAC and EU, were set and revised. This recommendation was also suitable to apply to the process of setting maximum residue levels in coffee, cocoa, chrysanthemum and other Chinese herbs. The above-mentioned principle regarding the establishment of maximum residue levels should be considered by the tea industry when applying pesticides to tea plants. In China, to ensure the safety of tea drinkers, it is recommended that some pesticides are not used in tea production including imidacloprid, acetamiprid, thiacloprid, dinotefuran and dimethoate due to their high water solubility.

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