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REVIEW ARTICLE

Exploring pioneering efforts in tea breeding and genetic transformation and designing driving innovative strategies for better brewing

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Abstract

Tea is the second most consumed drink in the world, following water. It is known for its aromatic allure, sense of refreshment, medicinal values and also nutritional properties, including antioxidants, anti-ageing, anti-inflammatory and anti-microbial nature. Tea breeding plays a pivotal role in the development of superior lines which can thrive in wider environmental conditions. However, conventional methods met with limited success, biotechnological interventions have shown their potential to evolve superior cultivars within a short span of time. Plant tissue culture technology allows for *in vitro* propagation that enables mass multiplication of uniform, elite clones with desirable traits besides serving as basic requirement for all the transgenic endeavours. Advances in omics technologies, coupled with advanced bioinformatics pipelines have led to the elucidation of key genes driving molecular events that confer increased tea yield and quality. Genetic transformation mediated by *Agrobacterium,* particle bombardment and CRISPR-Cas9 facilitate the production of transgenic tea with desirable traits. Inter-disciplinary collaboration among breeders, geneticists, agronomists and biotechnologists holds great promise in addressing the demands of consumers and overcoming the existing and emerging challenges posed by varied biotic and abiotic stress.

Keywords

decaffeinated tea; genetic transformation; genomics; molecular breeding; plant tissue culture; Tea.

Abbreviations: Ca- Calcium, Mg- Magnesium, N- Nitrogen, K- Potassium, Cu- Copper, Zn- Zinc, B- Boron, Fe- Iron, $ZnSO_4$- Zinc sulphate, $CuSO_4$- Copper sulphate, DNA- Deoxyribo Nucleic Acid, PVP- soluble Polyvinyl-Pyrolidone, PVPP- insoluble Polyvinyl-Pyrolidone, MS- Murashige and Skoog, BAP- 6- Benzyl Amino Purine, IAA- Indole-3-Acetic Acid, IBA- Indole-4-Butryic Acid, TDZ- Thidiazuron, 2,4 D- 2,4- Dichloro phenoxy Acetic Acid, PBOA- Phenylboronic acid, NAA- Naphthalene Acetic Acid, CM, GA- Gibberlic Acid, ABA- Abscisic acid, *GUS*- β glucuronidase, PCR- Polymerase Chain Reaction, qRT-PCR- Real-Time Polymerase Chain Reaction, YEB- Yeast Extract Beef, T-DNA- Transfer DNA, $CaCl_2$- Calcium chloride, NaCl- Sodium Chloride, HPLC- High-Performance Liquid Chromatography.

Introduction

Tea (*Camellia sinensis* L., family Theaceae), whose leaves are processed to obtain the infusion, is one of the oldest non-alcoholic beverages consumed globally. It is

a cross-pollinated, perennial, evergreen crop predominantly grown in tropical and sub-tropical climatic conditions (1). They are profoundly known for their rich aroma, flavour and taste. They are also known to possess several medicinal, pharmacological, therapeutic and nutritional properties (2).

Globally, China contributes to 75 % of total tea production accounting for an annual yield of 3.1 million tonnes. While India contributes around 1.33 million tonnes. Over the last decade, the global consumption of tea has witnessed an increased growth of 3.5 % and it is expected for an annual increase of 4.9 %. India's production is anticipated to rise by 7.5 % by 2027, reaching 3.6 million tonnes. The tea industry plays a crucial role in supporting the livelihoods of over 35 million individuals, contributing to poverty alleviation, nutritional security and the development of rural and hilly regions, particularly by empowering small-scale farmers (https://www.fao.org/). A major portion of Indian tea is exported to Russia, the United States of America, Iran, United Arab Emirates, Iraq and Poland and registered an export of 200.79 million kg valued at Rs 5415.78 crores during 2021-22 (https://www.teaboard.gov.in/). As the largest consumer of tea in the world, production and consumption of tea in India are expected to grow annually by 2.3 % and 3.5 % respectively. (https://www.fao.org/3/cc0238en/cc0238en.pdf).

2. Benefits and Pitfalls of Tea Consumption

There is a growing attention on the consumption of tea, due to the diverse pharmacological benefits. The beverage comprises vitamins (B, C, E), minerals (Ca, Mg), amino acids (theanine, glutamic acid), catechin (Epigallocatechin-3-gallate), caffeine, polyphenols (flavonoids), all of which have significant health benefits. For instance, tea polyphenols are known to enhance both mental alertness and immune response. They also exhibit properties like antioxidant, anti-bacterial, anti-viral, anti-inflammatory, anti-allergic and anti-ageing (3). Catechins are the predominant polyphenols in tea which possess several health benefits like anti-diabetic, anti-radiation, anti-microbial properties (4), anti-hypertensive and anti-arteriosclerosis (5). Caffeine, theophylline and theobromine are naturally occurring compounds which enhance cognitive performance, increase alertness and stimulate the central nervous system (6). L-theanine, a non-protein amino acid has been found to improve learning ability, promote relaxation, suppress high blood pressure and decrease levels of anxiety (7). In addition to its flavor and fragrance, *Camellia* seed oil is widely utilized for culinary applications.

However, overconsumption of tea has few ill effects. This is primarily due to the presence of caffeine (11 mg/100 g of tea) (https://fdc.nal.usda.gov/fdc-app.html#/food-details/174873/nutrients). The effects of caffeine consumption in the long term have detrimental effects on health. Overconsumption causes ill effects in gastrointestinal systems as well as elevated respiration, nervousness, irritability, insomnia, nutrient mal-absorption, etc. (8). In order to safeguard the health of both the fetus and the nursing infant, pregnant women and lactating mothers should limit their consumption of caffeine. This precaution is necessary as caffeine has the ability to permeate body water, enabling it to reach the developing fetus through the placenta. Moreover, caffeine has been found in the breast milk of nursing mothers, underscoring the need for restriction in this population. It has been suggested that for the healthy adult population, the acute dose of daily caffeine intake is estimated to be 150- 200 mg/kg (9).

3. Demand for Tea and Constrains to its Production

The global tea market is diverse and robust. The demand for tea production has increased substantially in recent years due to the growing concern for health and well-being among individuals and also the preference of consumers for premium and special tea. The increased consumption can be related to its diverse health benefits and is projected to drive significant demand in the future. Albeit the rapid growth in tea production, several challenges constrain the cultivation. Tea plantations are affected by several pests and diseases. For example, fungal infection causes loss in both quality and yield. Foliar diseases (including blister blight, grey blight, brown blight, twig dieback, root rot and stem cankers) affect the harvest directly while stem and root diseases affect the survival of the plants (10). In addition, it has geographical limitations due to the requirement of unique agro-climatic conditions. For example, in India, the ideal temperature for maximum tea production is 28 ± 2 °C along with uniform rainfall and a minimum daylight of 11 hours and 15 min which are required for sufficient flush growth. As they are dormant in the winter, the productive season is limited to a span of 6 to 7 months (11).

Camelia sinensis var. *sinensis* is prone to erratic climatic conditions, affecting both yield and quality of produce. Global warming tends to increase the incidence of new diseases and alter host-pathogen interactions and enzyme activity. Factors such as species, altitude, climate, pruning and fertilizer application affect the tea quality, especially levels of flavanols present in tea. With an increase in temperature, levels of catechins and caffeine are reduced. Higher precipitation alters the composition of aromatics and metabolites, while stress conditions reduce stomatal conductance thereby reducing the rate of photosynthesis and respiration. As tea is grown in monoculture, it has a negative impact on chemical properties like soil acidification, nutrient imbalance and also deterioration of microbial activity. By 2050, it is estimated that tea production in Assam might be reduced by 40% if necessary actions are not undertaken (12). Thus, adapting suitable agronomic and protective measures is essential for overcoming limitations in tea production to promote sustainability, combat climate resilience and social well-being of tea growers.

4. Agronomic ways to improve tea production and their lacunas

Among these practices, irrigation has demonstrated a positive impact on yield and is effective in disease control. Specifically, sprinkler irrigation has been observed to significantly reduce leaf temperature, which in turn enhances the photosynthetic rate. This practice has not only led to a greater number of harvestable shoots but also promotes the growth of finer roots. However, drip irrigation has emerged as a more successful micro-irrigation system, offering the advantage of enabling fertigation, which subsequently lowers labour costs associated with manual fertilization. Additionally, the implementation of raised beds has improved the overall efficiency of irrigation. In terms of drought mitigation, a combination of high-shade

trees, such as *Glyricidia* sp. and drought-resistant cultivars proved to be an effective strategy (13). Deficiency in N, K, Ca, Mg, Cu and Zn can be corrected by foliar spray of 1 % Zn/$CuSO_4$ solution mixed with 1 % urea and a wetter to initiate the active growth of the flush 2-3 times a year. Whereas, deficiencies in B, Fe, Cu and Zn can be addressed by applying foliar sprays of Boric Acid (0.1 %) or 1 % Iron, Copper and Zinc Sulphate or applying solubor at 5-10 kg/ha or by using sulfates of Fe, Cu and Zn at 5-10 kg/ha to the soil (14). Furthermore, the excessive use of chemical fertilizers has a detrimental environmental effect contributing to soil degradation and contamination of water bodies which threatens sustainability, ecological equilibrium and human health. It is imperative for farmers to ascertain the appropriate quantities, timing and frequency of fertilizer applications for maximum crop growth and yield. Consequently, the adoption of organic fertilizers, organic mulches, compost and biochar could be promising alternatives to chemical fertilizers. They are also known to adjust soil acidity, restore soil health and improve both yield and quality. It is also notable that both intercropping and agroforestry practices may increase the overall retention of nutrients and ecosystem productivity. Enhancing productivity can also be achieved through various methods, including proper plucking and pruning techniques. Integrated pest and disease management practices can also be advocated as they exhibit minimal side effects.

Thus, the importance of soil health and sustainable production should be considered and necessary management practices should be effectively followed to improve the yield and quality of produce, ultimately benefiting the economic stability of tea growers. Conversely, the development of an efficient tea clone that is responsive to all the inputs could serve as an effective and affordable strategy to overcome the existing production lacunas.

5. Protective measures followed in tea plantation

Pests and diseases pose a considerable threat to tea plantations, leading to annual crop losses ranging from 10-15 % with a potential total loss in severe cases. Excessive use of pesticides might develop resistance in pest, thereby adversely affecting the non-target species. Pesticide residuals on tea leaves may subsequently enter the food chain. Therefore, there is a need to confront the challenges associated with pests and diseases to foster sustainable production. Integrated Pest Management includes cultural, physical and mechanical practices along with the use of biological control agents, all aimed at reducing the reliance on chemical fertilizers. Cultural control methods are widely practiced due to their cost-effectiveness, efficiency and safety. It comprises plucking, pruning, shade regulation, field sanitation and use of trap crops. Physical control strategies employ devices to reduce the pest populations through direct intervention. It includes techniques like hot water treatment, soil solarisation, etc. Mechanical control methods contribute to pest suppression. For the effective destruction of termitaria, practices like mound digging, the use of cocktail mixture and food traps were utilized. Biological control measures focus on preserving the natural enemies within the ecosystem. It includes the use of predators, parasitoids sex pheromones, etc. Though the use of pesticides is detrimental, they play an important role in pest control. While the use of pesticides can be harmful, they remain a crucial component of pest management. Thus, careful consideration of the appropriate pesticide selection, dosage, timing and application methods is essential for effective pest control (https://www.teaboard.gov.in/pdf/PPC_Version_11_pdf3809.pdf).

Biopesticides in conjunction with less toxic chemical pesticides are proven to be an effective pest control. Microbial Biological Control Agents (MBCA) like *Bacillus* sp., *Pseudomonas* sp. and *Trichoderma* sp. demonstrate greater efficacy when utilized in consortium rather than as isolated antagonists. Strict quarantine measures are implemented to prevent the introduction of foreign pests and pathogens. It is also essential to strictly adhere to the norms formulated by the Government regarding pesticide application (15). The implementation of training and demonstration initiatives to sensitize farmers alongside promoting the use of green compost and manure and strict proctoring of agrochemical inputs at the market can play a pivotal role in agricultural advancements. Weather-based forecasting models, GIS technology and remote sensing for pest control are also under development. The global tea industry currently faces the challenge of producing pesticide-free, good-quality tea at a reasonable price while ensuring sustainable practices. To address these issues, breeding technologies can be employed to alter the genetic framework of tea plants, resulting in cultivars that exhibit improved characteristics, such as resistance to tea mosquito bugs and anthracnose, thus mitigating field-related challenges. Thus, the ultimate aim of tea breeding is to enhance crop performance, increase yield and revenue, promote biodiversity and support sustainable agricultural practices.

6. Breeding in tea

Tea, a cross-pollinated crop exhibits significant genetic variability among its offspring. This inherent heterogeneity presents considerable challenges in the development of pure lines, making the process both time-intensive and practical difficulties. Therefore, a better understanding of the selection of parental groups and their corresponding genetic constitution can speed up the breeding program (16).

Conventional or classical breeding practices comprise selection, hand-pollination, mutational breeding, polyploid breeding, controlled hybridization, etc. In tea, conventional breeding has been employed since 1939 for the development of mutants and other genetic resources. The process of controlled hybridization facilitates the production of hybrid seed varieties yielding high-quality cultivars by crossing the selected parents. Polyploidy breeding and mutational breeding primarily focus on enhancing yield and genetic diversity respectively. Traits like anthocyanin pigmentation and other quality characteristics in Darjeeling tea might be introduced from its wild relatives (17). A natural cross between *C. taliensis* and *C. sinensis* led to the development of a cultivar distinguished by low levels of caffeine with increased theobromine content. Genetic analysis revealed that a single recessive gene locus is associated with the lack of caffeine, suggesting that this hybrid could serve as a significant resource for the introduction of caffeine-free traits into cultivated tea varieties through controlled breeding practices.

6.1 Limitations of conventional tea breeding

Conventional tea breeding has its drawbacks which are accounted by the perennial nature of the crop, long juvenile period, high inbreeding depression, self-incompatibility, short flowering time (2- 3 months), clonal differences of flowering time, long duration for seed maturation (12-18 months), low success rate of hand pollination, lack of clear selection criteria, lack of tolerant mutants to biotic and abiotic stress, intensive labour demanding and time-consuming. On the whole, the entire process requires more than 2 decades right from selection to release of cultivar for commercial utility (1). Ultimately, there is an urgent need for the acceleration of the tea breeding program to improve the genetic makeup of the crop by incorporating novel genes in an elite cultivar for sustainable and enhanced production.

6.2 Molecular Tea Breeding

Quantitative traits exhibit continuous variation, necessitating the development of tools to categorize them into distinct classes. This requirement has led to the introduction of markers, which can be characterized as tags that are closely associated with the trait of interest. Molecular markers present significant advantages compared to traditional markers, such as morphological and biochemical markers. Unlike these conventional markers, molecular markers remain unaffected by environmental influences, epistatic interactions and pleiotropic effects, ensuring their stable expression across different conditions (18). Molecular markers, Marker Assisted Selection and DNA Fingerprinting are useful for identifying superior plant varieties and are found to strengthen conventional breeding strategies. Recently released commercial tea cultivars with their improved characteristics have been listed in (Table 1).

6.2.1 Molecular markers in tea

Similarly, Marker Assisted Selection (MAS) assists in the genetic improvement of perennials like tea in comparison with annuals. Initially, Random Amplified Polymorphic DNAs (RAPD) and ISSR have been employed in tea (19). However, after the introduction of Simple Sequence Repeats (SSR), they were the frequently used marker class; For example, 689 functionally relevant SSRs were successfully validated and they were related to aroma, quality and stress tolerance genes (20). In another study, 2554 single nucleotide polymorphism (SNP) within Nitrogen Use Efficiency- related genes were identified and they were converted to Kompetitive Allele Specific PCR (KASP) markers. These markers were used in 35 tea germplasm to assess the genetic analysis (21). Particularly, marker *EST-SSR073* was associated with resistance to Blister Blight (22), *CsCHIT* was associated with Pathogenesis-related protein production (23) and *OPW-03725*, *OPT-01625* and *OPG-11750* were associated with resistance against tea mosquito bug (24).

6.2.2 Molecular breeding coupled with omics studies

Markers also play critical roles in novel strategies such as Genomic Selection, which is now considered the trendy tool for genetic improvement and evaluation programs in tea. Genomic selection (GS) can increase genetic gain and shorten the breeding cycle in tea breeding programmes. Genomic Prediction and Genome Wide Association Studies (GWAS) are effective tools for the genetic improvement of tea quality-related metabolites, especially catechins and caffeine (16). A study involving 95 tea accessions of the Biluochun tea plant identified significant associations between SNPs and metabolites like GC, GCG and caffeine, revealing 4 potential candidate genes involved in the regulation of synthesis of tea-related metabolites (26). Integrated analysis by GP and GWAS

Table 1. List of commercial tea cultivars released and their unique features.

Cultivar	Year of release	Country	Method of improvement	Improved characteristics	Reference
TV26	1996	India	Molecular (Marker-Assisted Selection)	Improved flavor, pest resistance, high yield	Tea Research Association (TRA), Tocklai, 1996
UPASI-9	2008	India	Traditional (Clonal Selection)	High yield, improved resistance to drought	United Planters' Association of Southern India (UPASI), 2008
TRA T-383	2010	India	Traditional (Hybridization)	Improved quality, pest resistance (tea mosquito bug)	Tea Research Association (TRA), Tocklai, 2010
Kenya TRFK 6/8	2011	Kenya	Molecular (Marker-Assisted Breeding)	Disease resistance, improved leaf quality, high yield	Tea Research Foundation of Kenya (TRFK), 2011
TRA PM 7	2012	India	Traditional (Clonal Selection)	Improved quality, early maturing	Tea Research Association (TRA), Tocklai, 2012
TRFK 306/1	2013	Kenya	Molecular (Marker-Assisted Selection)	Drought resistance, high yield, improved leaf quality	Tea Research Foundation of Kenya (TRFK), 2013
TRA SK 17	2014	India	Traditional (Hybridization)	Pest resistance, better flavor, improved yield	Tea Research Association (TRA), Tocklai, 2014
UPASI-17	2015	India	Traditional (Clonal Selection)	Drought tolerance, pest resistance, high yield	United Planters' Association of Southern India (UPASI), 2015
TRA CK 25	2016	India	Traditional (Hybridization)	Pest resistance, improved flavor profile	Tea Research Association (TRA), Tocklai, 2016
TRI 2025	2017	Sri Lanka	Traditional (Hybridization)	Drought resistance, superior leaf quality	Tea Research Institute (TRI), Sri Lanka, 2017
TRFK 371/3	2018	Kenya	Traditional (Hybridization)	Frost tolerance, high yield, drought resistance	Tea Research Foundation of Kenya (TRFK), 2018
Assamica T-9	2018	India	Traditional (Clonal Selection)	High caffeine content, pest resistance	Assam Agricultural University, 2018
TRFK 430/90	2019	Kenya	Traditional (Hybridization)	High yield, pest resistance, drought tolerance	Tea Research Foundation of Kenya (TRFK), 2019
Jorhat 2020	2020	India	Traditional (Clonal Selection)	High yield, pest resistance, mechanization-friendly	Assam Agricultural University, 2020
Zhejiang 2021	2021	China	Traditional (Clonal Selection)	Cold tolerance, early budding, high catechin content	Zhejiang Tea Research Institute, 2021

for genetic improvement of tea quality-related metabolites using genome-wide SNPs from RAD-seq data was also performed by (27). Despite these progresses, introgressing the better traits through marker-assisted breeding is yet to be demonstrated in tea. To widen the available germplasm of tea, future breeding strategies should aim to incorporate multiple disease and pest-resistant alleles and also to develop adapted tea cultivars with low caffeine content. To this end, strategies like inter-specific hybridization along with marker-aided introgression can be adopted in tea breeding (28).

There has been increasing demand to widen the breeding methodologies to improve the genetic makeup of tea plants as well as to reduce the period of breeding. Furthermore, a significant proportion of tea plantations are likely derived from seeds, resulting in considerable genetic variability. To address these challenges, advancements in biotechnological research have introduced more effective production techniques that also focus on cost reduction. These methods are deemed reliable since the molecular traits remain stable regardless of environmental fluctuations. Key approaches include micropropagation, somaclonal variation via tissue culture, cybrid generation through somatic hybridization and the creation of transgenic plants through genetic engineering (29).

7. Tissue Culture Studies in Tea

Vegetative propagation facilitates the cloning of genetically superior material, with the ability to produce desirable characteristics such as enhanced quality, increased yield, the development of homogeneous populations and new cultivars which are resilient to both biotic and abiotic stresses. Nonetheless, challenges such as poor root formation and the seasonal constraints on rooting for certain clones restrict the year-round availability of planting materials. Consequently, the implementation of micropropagation techniques is essential to address the limitations associated with traditional propagation methods (17).

7.1 Micropropagation of tea

A successful micropropagation technology is directed towards commercial utilization. *In-vitro* propagation of tea depends on the type and nature of the explant used. The most commonly used explants are nodal segments, shoot tips and leaves. Research was also conducted with zygotic embryos and immature and mature cotyledons as well. While callus formation was observed with varying degrees of success depending on the explant type, the regeneration of the whole plant has not been consistent. The major problems faced in the micropropagation of tea include the phenolic exudation from explants and the risk of microbial contamination from both the explant and culture medium as well. Cut ends of the explant on enzymatic oxidation, releases toxic compounds which subsequently reduce the pH of the medium. Ascorbic acid and PVP were used to mitigate the browning of the explant (29).

7.1.1 Culture medium for tea micropropagation

The most commonly used media are typically full-strength or half-strength Murashige and Skoog salts, often with some modifications. Woody Plant Medium and Heller Medium are potential alternatives. Sucrose is the preferred carbon source over other sugar compounds like maltose, lactose, sucrose and galactose (30). To promote growth rates, hormones are added at different concentrations. UPASI 9, a popular variety grown in the Nilgiris district of Tamil Nadu, is known for its superior quality and productivity. Nodal explants from UPASI 9 were cultured on MS + BA (5 mg/L) + CM (10 %) for initiation of callus. The most preferred plant growth hormone for shoot initiation and proliferation includes BA and IBA. TDZ is found to be a potent cytokinin which results in an increased proliferation rate (31). MS + GA_3 (1 mg/L) + BAP (0.5 mg/L) was the shoot multiplication medium used for the proliferation of UPASI- 9 (32). Reports suggest that half-strength MS is more suitable than full MS for shoot proliferation.

Efforts have been taken to establish liquid cultures due to their cost-effectiveness and potential for increasing the numbers and as progress towards automation. Notable success has been witnessed when *in vitro* cultures were used as the explants in liquid MS medium supplemented with 1.1 mg TDZ. It proved to be effective for both initiation and proliferation. It was determined that a 20 mL static liquid medium with a subculture period of 6 to 8 weeks was found to be labour and cost-effective when compared to the existing protocol (33).

7.1.2 Hardening

The critical phase of micropropagation is characterized by the acclimatization of the *in vitro*-grown plantlets. Various factors like temperature, humidity, light and dark period, light intensity, soil pH, CO_2 and microclimatic conditions influence the process. *In vitro* rooting efficiency depends on genotypic variation among the cultivars. The best results were observed when the concentration of MS salts was reduced to either half or quarter strength. Roots treated with IBA were found to be long and fibrous (29). The rooting medium comprising MS media supplemented with IAA (1 mg/L) exhibited an 18 % rooting response for UPASI-9 (32). Furthermore, dipping the roots in auxin followed by dark treatment significantly improves the rooting efficiency. When shoots were treated with IBA for 30 min prior to being placed in a sealed jar containing sand, a survival rate of approximately 88 % was achieved. Rooting can also be achieved in low auxin media treated for a long period or auxin shock at high concentration and subsequent transfer to a medium that is auxin-free (1). It was also discussed that low pH (about 4.5- 4.6) and low light conditions favoured root induction. Thus, it is evident that the need for the optimization of media for individual cultivars is crucial for effective micropropagation.

In vitro-grown plants can be transitioned to an *ex vitro* rooting process. *In vitro* rooted tea plants of 5- 8 cm were subsequently transferred to pots containing fumigated soil. They were then placed in a humid chamber for 10 days during which they gradually acclimatized to field conditions. Potting mixture of a 1: 1 ratio of peat and soil under high humidity gave optimum results (34). Biological hardening is the emerging concept for hardening of micro propagated plants. It involves the use of microbial cultures to overcome the effect of microbes in the rooting media.

7.2 Somatic embryogenesis

Indirect organogenesis in *Camellia* sp. presents considerable challenges when utilizing the vegetative part of the plant (35).

Somatic embryogenesis is considered a valuable tool for genetic improvement, playing a crucial role in the production of somaclonal variants. It integrates well with advanced biotechnological approaches like gene cloning and transformation. Despite the difficulties associated with woody species, the somatic embryos produced exhibit bipolar characteristics, making this approach a potentially more cost-effective alternative to micropropagation techniques like micro cuttings (36).

Folded immature leaves of *in vitro*-grown plants were chosen as explants for somatic embryogenesis (37). They were cultured on basal MS medium supplemented with 5 mg/L 2, 4 D. After 6 weeks of culturing, the tissues were subsequently transferred to liquid MS medium containing 0.5 mg/L 2, 4 D. Somatic embryos were formed directly on the tips of leaves and midribs or indirectly from the basal region of the petiole, with a success rate of 10 %. The germination of these somatic embryos to secondary embryos was carried out in MS medium containing 10 mg/L BA and 0.5 mg/L IBA leading to further development into complete regenerated plantlets. The number of adventitious embryos increased when half-split cotyledons of the 'Yabukita' variety were cultured on basal MS medium (38). Increased frequency of somatic embryo induction of 59 % and 56 % was achieved on MS medium supplemented with 1 µM PBOA + 0.5 µM BA and 1 µM PBAO + 0.5 µM Kinetin respectively (39). While 2, 4-D was effective, other auxins did not promote rhizogenesis. These plantlets, when subjected to a liquid MS medium with 1 µM Brassin, 1 µM IAN and 10 µM Phloroglucinol for a duration of 15 days, exhibited a significant frequency of lateral root formation and plant recovery. After 200 days of culture initiation, the plants were transferred to red soil with a pH of 5.0. nodal explants of TRI 2025 in MS medium enriched with 1 mg/L of 2, 4- D and kinetin each gave rise to callus and also characterized with embryoid-like structures (40). Roots developed in callus sub-cultured in a medium containing 2 mg/L IBA and 10 mg/L of BAP in full-strength MS salts. The Temporary Immersion System was reported for direct somatic embryogenesis from cotyledon of the 'Yabukita' variety (41). Cotyledons were cultured on half-strength MS medium containing 2 mg/L of BAP increased the pro-embryogenic mass and their development and maturation were enhanced by the use of kinetin and ABA at 0.1 mg/L each. Germinated embryos with distinct roots and shoots were obtained with a success frequency of 30 % and eventually, they were acclimatized in a pre-sterilized 3:1 soil and sand mixture at the screen house. *In vitro* regeneration of tea via somatic embryogenesis from immature cotyledon tissues was carried out with a high rate of embryo survival (87.3 %) and the maximum rate of plant regeneration (58.3 %) was achieved in basal MS media supplemented with 1.5 mg/L PBOA and 1 mg/L kinetin. In addition, increased concentration of kinetin resulted in the formation of somatic embryos from immature cotyledon (42).

7.3 Other tissue culture methods

Protoplast culture is an emerging technology to explore cell biology, cell division, differentiation, regeneration and plant physiology. Under favourable conditions, protoplast can regenerate into a whole plant. Successful protoplast culture depends on quality protoplast, which in turn depends on the method of isolation such as mechanical or enzymatic and the availability of cell suspension culture. The first tea cell suspension culture was isolated from the leaves and petals of tea (43). PVPP was added to the isolating medium to overcome polyphenol oxidation and endophytic growth from *in vitro* grown leaves. A viable protoplast (87 % yield) was obtained in the 'Yabukita' variety (44).

Somatic hybridization also has tremendous potential for the production of low-caffeine tea by fusing the protoplast of aroma-rich and caffeine-free tea with cultivated tea (1). Anther culture has also been employed in tea to produce homozygous diploid plants, with successful development observed from the anthers of 'Fuyun No 7' on the N6 medium (45). Despite several attempts to regenerate haploid plants, progress has often been impeded at the micro-calli stage.

The commercial application of micropropagation in tea remains unrealized. This is primarily due to the challenges posed by the lack of juvenility and the tap root system in *in vitro* cultures, which renders micropropagated plants more vulnerable to environmental stress. Conversely, plantlets obtained from somatic embryos exhibit tap roots and thrive better under environmental conditions. Despite these advantages, efforts are still necessary for the commercialization of somatic embryogenesis, particularly due to the complex nature of tea seeds. Given the limited success of alternative tissue culture methods, there is an urgent need for tea breeders to enhance production capabilities to satisfy growing market demands. Thus, gene transfer through biotechnological approaches appears to be a promising avenue for future exploration (17).

8. Genetic transformation in Tea

8.1 Transformation mediated by *Agrobacterium tumefaciens*

Transformation was performed in the embryogenic callus of Tingamira Normal, a variety of *C. sinensis* var. *assamica* using *A. tumefaciens* strain EHA105 containing pMOG410 vector system. To the *GUS* gene, *caffeine synthase* gene (*TCS 1*) or anti gene (As*TCS 1*) was cloned. Co-cultivation was performed in either dark or light conditions for 3 days in MS medium supplemented with 0.5 mg/L 2, 4-D. They were transferred to the same medium along with 300 mg/L carbenicillin and 50 mg/L of kanamycin without acetosyringone. Subculturing was done on medium with carbenicillin, kanamycin, 2 mg/L IBA, 1 mg/L GA_1 and 4 mg/L BA. Embryos developed into cotyledons and the event of transformation was confirmed by performing PCR (85 % efficiency) and RT-PCR. Thus, the transformed tea plant would be expected to be caffeine-free (46, 47).

Transformation of the leaf was carried out using *Agrobacterium* strain LBA4404 carrying pBI121 binary vector. Leaves were co-cultivated on MS media supplemented with 10 mg/L BA and 0.5 mg/L IBA in the dark for 2 days. To kill the excess bacterial growth, they were treated with 500 mg/L of carbenicillin. They were transferred to selective media containing 500 mg/L carbenicillin and 200 mg/L of kanamycin. Cultures were maintained at 25 °C with light and dark periods of 16 h and 8 h respectively. Three out of five resistant, transgenic calli were confirmed by *GUS* assay, PCR and Southern blot. The remaining 2 calli could not be confirmed if

they are transgenic because of lack of amplification (48, 49).

A successful genetic transformation was reported in Kangra Jat. The somatic embryos were co-cultivated with *Agrobacterial* suspension for 20 min. Followed by co-cultivation, somatic embryos were transferred to a medium with bactericidal antibiotics like cephalexin and carbenicillin for 15 days. The appearance of blue spots on embryos after 48 h indicated the *GUS* activity and thus confirmed transformation. They were then transferred to the same media with any one of the bactericidal antibiotics along with kanamycin. After consistent subculturing, *GUS*-positive, kanamycin-resistant transgenic embryos were transferred to multiplication and germination medium for shoot proliferation. Finally, transgenic shoots were micrografted on the same cultivar. They also found that higher transformation efficiency was obtained without pre-culturing with optimum bacterial density OD_{600} value 0.6 and the pH of the co-cultivation medium being 5.6. The 5 plants obtained were characterized on a molecular level using PCR and Southern Hybridization (55).

Few have attempted (51-53) *Agrobacterium* mediated transformation in tea with minimal success. *Bt* gene expression vector was constructed and *Agrobacterium*-mediated transformation was performed using leaf discs and calli derived from the leaf or stem or seed of several cultivars. *A. tumefaciens* strains LBA4404, EHA105 and *Agrobacterium rhizogenes* pRil5834 in conjugation with recombinant pCAMBIA2301 containing *Bt* gene was chosen as vector. Zhenong 23 and Longjingchangye incubated with *A. tumefac*ines EHA 105 and *A. rhizogenes* pRil5834 confirmed the *GUS* assay. A threshold level of hygromycin (20 µg/mL) and kanamycin (50 µg/mL) as selection agents was effective for callus and leaf respectively. It was discovered that the high phenol concentration in tea contributed to the explant browning which caused the death of the explant. The use of antioxidants or pre-treatment of explant with PVP or half-strength MS medium was recommended. It was deduced that the explant when pre-cultured in the appropriate medium for 3 days and co-cultivated at 25 °C for 3 days gave the best results (54).

Acetosyringone, a principal inducer was ineffective in *Agrobacterium* infection in tea. This is because the oxidation of polyphenols led to the production of quinone. Research revealed that caffeine could serve as a substitute for acetosyringone as it reduces the oxidation rate of polyphenols (33). Some have studied (55, 56) the optimal conditions for bacterial transformation in tea. The effect of leaf characteristics on *Agrobacterium* infection was explored (57). Leaves of TV1, UPASI-9 and Kangra Jat showed 75 % infection due to their high degree of wettability whereas leaves of UPASI-10 and St-499 showed 50 % infection with a relatively lower degree of wettability, high trichome density and lower wax content. Low phenol content (200 mg/L) promoted the growth, but high concentrations of polyphenol (above 250 mg/L) were reported to be lethal. Using disarmed EHA 105 strain of *Agrobacterium* carrying p35*GUS*INT as a vector, leaf samples of Kangra Jat were submerged in bacterial culture for 20 min and then transferred to co-cultivation media with autoclaved L-glutamine (pH 5.4) for 5 days at 19 °C. They were washed with liquid basal MS medium supplemented with 1.5 mg/L of 2, 4- D and 400 µg/mL cefotaxime to remove excess bacteria. After blotting, the explants were transferred to a selection medium which had 50 µg/mL of hygromycin and 10 mg/L of 2, 4- D for a week and taken to a selection medium which is cefotaxime free. Hygromycin-resistant calli were transferred to a shooting medium. Putative transformants were confirmed by PCR (60 %) and Southern Hybridization. They also stated that L-glutamine induced the expression of *vir* genes and has the ability to overcome the bacterial effects of polyphenols (58-62).

Compounds like ascorbic acid, cysteine, charcoal and PVP effectively absorbed polyphenols, however, they failed to infect the tea leaves. The successful infection of *Agrobacterium* was observed in cultivars (UPASI 9, UPASI 10, Stock 449, Kangra Jat, Tocklai Variety 1) when filter-sterilized L-glutamine and L- glutamic acid (0.5 g/L) were present in the co-cultivation medium. These compounds bind with quinone derivatives to form harmless compounds that ensure the viability of healthy explants. Aseptic leaf explants were infected with the EHA 105 strain harboring pBin19 derivative for 20 min. After blot drying, they were co-cultivated in the dark for 5 days. Overgrown bacteria were washed off with liquid MS medium consisting of 250 mg/mL cephalexin and 250 µg/mL carbenicillin. To initiate callus, they were transferred to basal MS media supplemented with 250 mg/mL of cephalexin and 10 mg/mL of 2, 4- D. Selection media had 30 µg/mL of hygromycin. Calli were then transferred to shoot regeneration medium containing MS medium with 1 mg/mL of IBA and 2 mg/mL of BA. Transformants were successfully obtained for Kangra Jat, Tocklai Variety 1 and UPASI 9 and were confirmed by PCR and Dot Blot analysis (63). A study on the effects of catechins on the transformation of tea was conducted. GV3101 strain of *A. tumefaciens* containing pBI121 vector was utilized for the transformation of tea leaves, stem and hypocotyl. Catechin was added to the LB medium whereas LB medium without catechin served as control. Co-cultivation was done for 3 days in the dark and transferred to a half-strength MS medium containing 2,4-D, Kinetin, cefotaxime and kanamycin for callus induction. No *GUS* activity was reported in leaves infected with catechin-treated *Agrobacterium* whereas 10 % of leaves infected with catechins untreated *Agrobacterium* showed *GUS* activity. Though stable *GUS* activity was recorded, no plantlets were regenerated (64-66).

Further optimization of efficient regeneration and transformation protocol was achieved in Longjing Changye (67). *A. tumefaciens* EHA105 and PS1aG-3 expression vector was used. Cotyledon callus was co-cultivated with bacterial solution (YEB + 0.2 mg/L IBA + 150 µmol/L acetosyringone) and transferred to the medium (Modified Eriksson Medium supplemented with 0.2 mg/L IBA, 100 mg/L cephalexin and 100 mg/L spectinomycin) in the dark. After strict sub-culturing, resistant cultures were transferred to a differentiation medium (Eriksson Medium supplemented with 0.1 mg/L IBA, 2 mg/L BAP and 100 mg/L spectinomycin) for about 30 days and finally the differentiated seedlings were transferred to rooting medium (1/2 Eriksson Medium supplemented with 0.2 mg/L IBA, 1 mg/L BAP and 100 mg/L spectinomycin). The peak transformation (46.7 %) was observed when the infection and co-cultivation time was 15 min and 3 days respectively. The

highest transformation ratio was observed when the concentration of acetosyringone was 150 µmol/L.

Agrobacterium-mediated co-transformation to confer resistance for blister blight in tea was reported for the first time. Somatic embryos of 'T-78' were chosen for transformation with the LBA4404 strain. Three transformation events were performed with constructs having *S. tuberosum* class I *chitinase* gene (pCAMBIA1301- Chi, transformation frequency of 2.20 %), mung bean *defensin* gene (pBI121- Def, transformation frequency of 2.39 %) and combination of both constructs transformation frequency of 3.41 %. Putative transformants and co-transformants were confirmed by PCR analysis. PCR-positive samples were further taken to transgene copy number analysis by real-time PCR (68, 69). Strains LBA4404, K599 and EHA105, ATCC15834 were resistant and susceptible to tea phenolics respectively (70, 71). Recently, *in-planta* transformation in tea was also reported (72). Apical and auxillary buds of tea seedlings were removed and wounded for bacterial infection. EHA105 strain carrying pCAMBIAB1301 was employed for the study. The wounds were injected with bacterial culture for 40 min using funnel-shaped sealing film, while double distilled water as a negative control. The cultures were incubated in the dark for 2 days at 28 °C. Cotton swabs dipped in hygromycin solution were placed over the incision and re-sealed again. Cultivation was conducted in the dark. After the resistant buds matured, they were transferred to light for the initiation of the leaf. They were subsequently relocated to the greenhouse to promote regeneration. A total of 299 resistant buds were confirmed by amplification of *GUS* and *hyg* genes *via* PCR. Sequencing of the PCR amplification product also confirmed the transgenic event. The study emphasized the importance of *in-planta* transformation studies in tea, as the steps were relatively simple, cost-effective with an average transformation rate of 4.55 %. A comprehensive study on *A. tumefaciens*-mediated transformation in tea has been illustrated in (Fig. 1).

The limitation associated with *Agrobacterium*-mediated transformation is the need for extensive washing of explants after co-cultivation to remove residual bacterial growth on the transformed explants. Improper washing can result in necrosis and eventual death of the explants (74). Additionally, the presence of polyphenols and their oxidation contribute to the mortality of the explant. Notably, catechin, a secondary metabolite found in tea has been shown to inhibit *Agrobacterium* growth (66).

8.2 Transformation mediated by *Agrobacterium rhizogenes*

The major reason for *A. rhizogenes*-mediated transformation lies in the ability to facilitate the production of secondary metabolites that are synthesized and stored in roots (74). This aids in the investigation of root functions and root-shoot interaction, encompassing the genes responsible for nutrient absorption, transport of hormones, symbiosis, pathogen interaction, etc. This approach allows for crop improvement while preserving the genetic integrity of the shoots. A brief overview of *A. rhizogenes*-mediated transformation is depicted in (Fig. 2).

Infection of *in vitro*-grown tea leaves with *A. rhizogenes* virulent strain A4 hairy roots was performed. The cut ends of the young leaves were dipped for 2 min in bacterial suspension and blot-dried. On MS medium supplemented with 1 mg NAA, the explants were co-cultivated with bacteria for 2 days at 25 °C in the dark. Successive subcultures were carried out in plain MS medium containing 1000 µg/mL Cephalexin at an interval of 15 days. Growth of roots was observed in the antibiotic-free medium and also presence of auxin had an important role in hairy root induction. White-coloured roots eventually turned green in the presence of continuous light. The study revealed

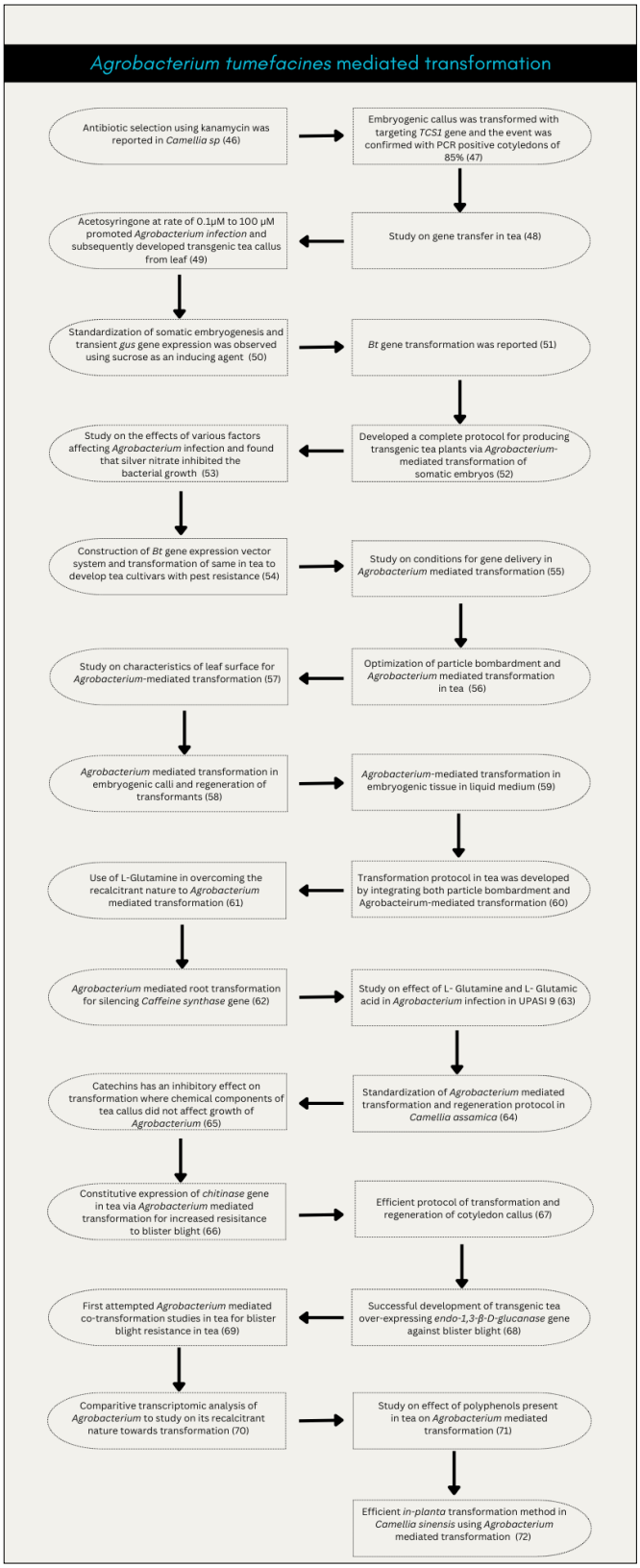

Fig. 1. Chronological works carried out in *Agrobacterium tumefaciens* mediated transformation in tea.

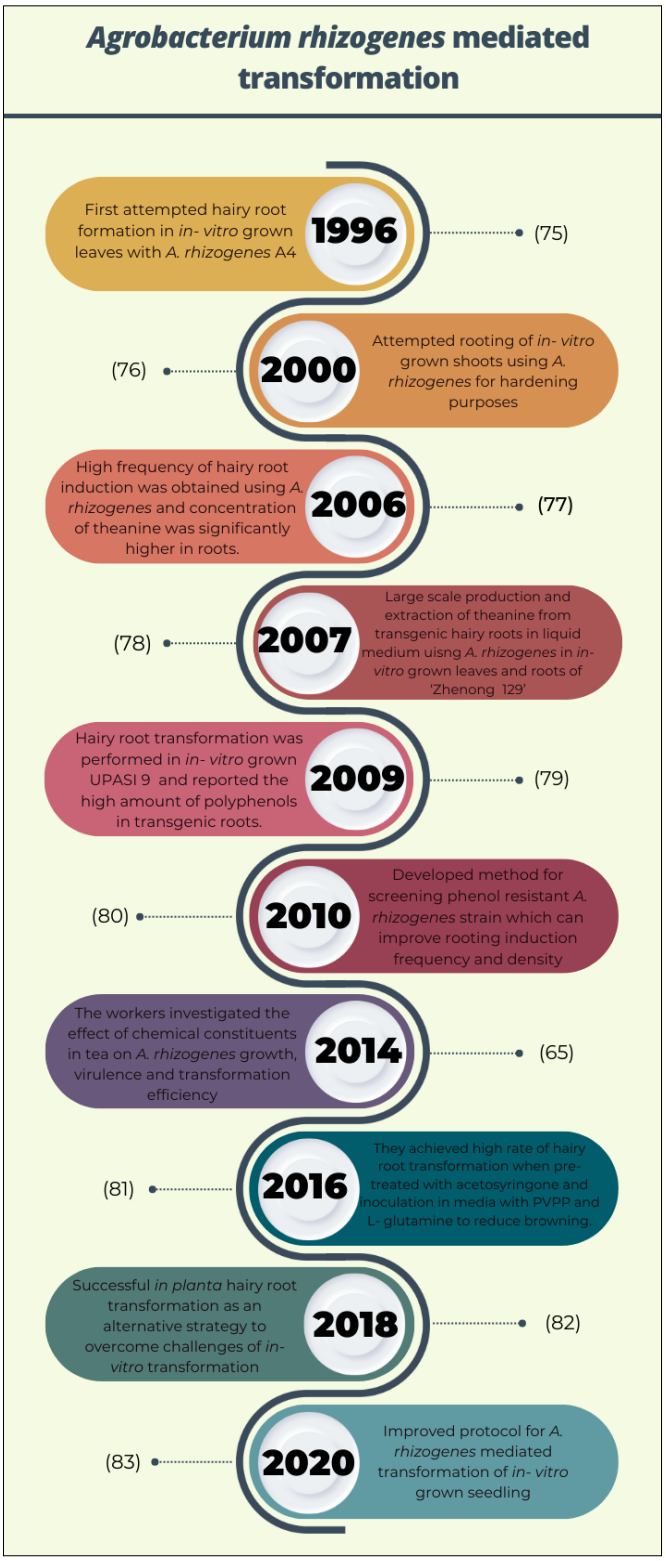

Fig. 2. Delineation of *Agrobacterium rhizogenes* mediated transformation in tea.

that the fully expanded leaves were not amenable to transformation due to excessive exudation of phenols (75). Rooting of *in vitro*-grown shoots was carried out using *A. rhizogenes*. Co- cultivation was performed in a liquid basal MS medium containing 5 mg/L of IBA and 100 mg/L of rifampicin. After 30 to 45 days of culture, growth of roots was observed and they were transferred to the nursery for hardening (76). Attempts on hairy root induction for production of secondary metabolities was performed by (77, 78). *In vitro*-grown leaf samples of UPASI 9 and strain MTCC532 were co-cultivated for 48 h at 25 ± 1 °C in the dark. They were subjected to liquid MS wash supplemented with 250 mg/L of cefotaxime. Sub-culturing was performed at an interval of 2 days. PCR analysis with *rolC* gene primers confirmed the event of transformation. Acetosyringone at the concentration of 300 µM/L, along with 3 % maltose proved to be effective in facilitating the transformation. The transformed calli when placed on a root induction medium enriched with IAA (5 mg/L), produced transgenic roots within 15 days. Notably, the transgenic roots exhibited an increased concentration of polyphenols, suggesting the potential need for commercialization (79, 80).

Strains ATCC15834 and K599 of *A. rhizogenes* were chosen for hairy root induction to study the effect of catechins on *Agrobacterium* infection. After 20 min of co-cultivation of tea stem, leaf and hypocotyl, they were transferred to a half-strength MS medium containing cefotaxime. The transgenic event was confirmed by the amplification of *auxI* gene by PCR. The K599 strain did not exhibit any hairy root development, whereas strain ATCC15834 induced transgenic root formation in cotyledon and hypocotyl explants within 20 days, whereas leaf and stem required 40 days for similar root induction (65). MS salts with 5 g/L sucrose and 0.1 g/L L-glutamine effectively mitigated explant browning, while a lower concentration of sucrose favoured maximal growth of *Agrobacterium*. The induction of hairy root with an efficiency of 16.7 % was achieved, when callus was pre-treated with *Agrobacterium* containing 150 µM acetosyringone, followed by inoculation in basal MS media enriched with 30 g/L sucrose, 5 g/L PVPP and 0.1 g/L of L-glutamine. Expression of *GUSA* and *cfp* (cyan fluorescent protein) genes in the transgenic roots confirmed the transformation (81). Successful *in-planta* hairy root transformation in Nong Kangzao was carried out using an A4 agropine strain of *A. rhizogenes* containing pBI121. *Agrobacterium* was cultivated on A4 media and a needle containing *Agrobacterium* at its tip was employed to puncture the hypocotyl region. The punctured region was smeared with *Agrobacterium* paste using an L rod. Subsequently, the plants were transferred to a humid chamber and subjected to 10 % suspension of A4 strain for 14 days. Following this, the growth chamber was placed in light and dark conditions for 16 and 8 h respectively. The plants were irrigated with 1 % MS medium and water for a period of 3 months. This procedure resulted in profuse hairy root formation (88.3 %). The PCR reaction was carried out to confirm the transgene integration and qRT-PCR to investigate the expression of root-inducing genes. The study emphasized that *in-planta* transformation presents a viable alternative to address the challenges encountered in *in-vitro* transformation (82).

Recently, an improved protocol for *A. rhizogenes*-mediated transformation was developed. Three-week-old *in vitro*-grown seedlings were aseptically transferred to screw-capped bottles containing A4 suspension. They were infected with a drop of A4 strain on a sterile needle for 8 min. After the removal of excess bacteria, they were co-cultivated at 27 °C in the dark for 3 days. The infected seedlings were transferred to a growth chamber for the development of hairy roots. Subsequently, roots were multiplied in the selection medium (MS + 100 mg/L cefotaxime + 100 mg/L kanamycin). Integration of T-DNA was confirmed by PCR amplification of *rolB, rolC* and *aux1* genes as well as *GUS* assay (83).

Various methods for developing transgenic tea encompass techniques such as particle bombardment, electroporation, microinjection and direct uptake of DNA. While these methodologies can be employed in plants that exhibit resistance to T-DNA mediated transformation, they tend to demonstrate a comparatively low efficiency in achieving successful transformation.

8.3 Transformation via Biolistics or Particle Gene Gun

In order to address the constraints associated with *Agrobacterium*-mediated transformation, biolistics has emerged as a prominent direct gene delivery method utilized for the transfer of foreign genes into plants. The technique typically involves coating of gold or tungsten particles with DNA, which are then propelled into plant tissues at high velocity. After bombardment, DNA elutes from the particle and becomes integrated into the genome or expressed transiently. The technology is successful in DNA delivery encompassing a wider host range. In tea, a gene gun has been used to engineer the biosynthetic pathways of aromatic compounds to enhance the flavor of the tea. Milestones in biolistics-mediated transformation are depicted in (Fig. 3).

DNA of plasmid p2k7 was coated with gold particles (1.5 -3 µm) using 1.0 M $CaCl_2$ and 0.1 M spermidine precipitation. Somatic embryos of tea were bombarded at a Helium pressure of 550 kPa using a Particle Inflow Gun (PIG) at a distance of 9.5 cm away from the site of DNA delivery. After 30-40 h of bombardment, the *GUS* histochemical assay revealed the highest transient expression (1085 blue spots/shot). It was also stated that mannitol pre-treatment did not have any effect on transformation (36). Transgenic tea plants were developed using *in vitro*-grown completely folded leaves as explant. Plasmid pRT99*GUS* was coated with gold particles at the rate of 1 µg/µL with a target distance of 6 cm and burst pressure 7584.23 kPa. Following the bombardment (PDS1000-He), they were transferred to basal MS medium containing 22.6 µM 2, 4-D and 1.71 µM kanamycin in the dark for 2 days and subsequently in light for 5 weeks. Putatively transformed leaves (7 out of 15 lines) were confirmed by *GUS* assay, PCR analysis and Southern Hybridization. For regeneration, the transformed leaf calli was transferred to MS medium supplemented with 8.88 µM BA and 1.71 µM kanamycin. The plantlets were transferred to pots containing sand: garden soil at a 1:1 ratio and maintained as tea bushes in polyhouse. The study concluded that transgenic tea lines produced more flower buds and fruits with a 7 % increased germination percentage than untransformed lines (84). A simple method for the production of transgenic tea was initially established (85) and another study (86) utilized plasmid pBinAR reconstructed with *osmotin* gene (gene codes for a cationic protein which confers salt tolerance) from *A. tumifaciens* GV2260 for somatic embryo transformation using gene gun. 0.6 µg of plasmid was coated on 0.5 mg gold particle (1 µm) and precipitated using $CaCl_2$ and spermidine. Micro projectiles were bombarded into globular somatic embryos using the PDS-1000/He system with pressure and target distance of 7.58 MPa and 9 cm respectively. After 48 h, the bombarded embryos were transferred to MS medium supplemented with half-strength nitrate, 300 mg/L potassium sulfate, 2 mg/L BAP and 0.2 mg/L of IAA for secondary embryogenesis. After 2 weeks, they were transferred to MS medium supplemented with half-strength nitrate, 0.2 mg/L BAP, 0.1 mg/L of IAA and 200 mg/L of kanamycin to screen the transformants. PCR was performed using *osmotin* gene-specific primers and a total of 674 positive transgenic lines were obtained out of 4500 lines. Randomly selected lines were subjected to Southern hybridization to confirm the transformation. Transgene expression was also confirmed by Northern Hybridization and RT-PCR. At 100 mM NaCl, the transgenic leaves remained green while the non-transformants bleached completely. Thus, successful transgenic tea lines with improved salt tolerance were developed (87). Recently, the optimal conditions for biolistic bombardment in tea were postulated. Somatic embryos of Tingamira Normal were pre-treated with 0.2-0.4 M mannitol in

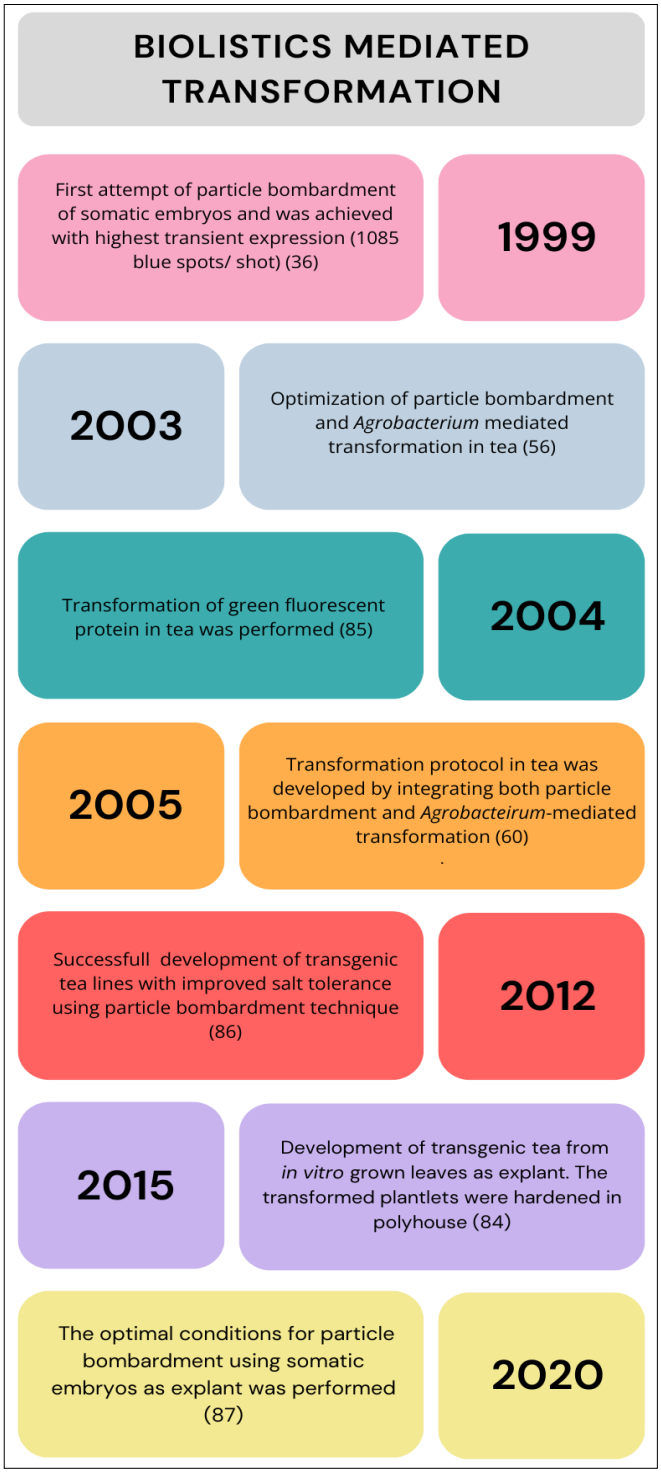

Fig. 3. Timeline of gene gun mediated transformation in tea.

hormone-free MS medium in the dark at 25 °C ± 2 °C overnight. Efficient transformation results were obtained when pre-treated embryos were bombarded (PDS-100/He) with conditions of burst pressure 1100 psi, gold particle diameter of 1.0 µm and target being at a distance of 6 cm. The appearance of blue spots in the *GUS* histochemical assay confirmed the transformation. The bombarded embryos were incubated at 25 °C ± 2 °C in the dark for 30 days in a mannitol-free MS medium for further development of somatic embryos (87).

9. Insights on Tea Caffeine Synthase (TCS)

The adverse effects of caffeine consumption have been discussed extensively. Therefore, measures to reduce the caffeine content can make it a more palatable option for consumers. Additionally, reducing the caffeine content in tea can increase the health benefits. For example, low-caffeine green tea has been shown to reduce stress levels and improve sleep quality in middle-aged individuals (88). In addition, the changes in the composition of other components, such as amino acids and flavonoids enhance the taste and quality of the tea. On an overall note, reducing caffeine content makes tea a safer and more enjoyable beverage for a wider range of individuals, while still retaining its health benefits.

The biotechnological approaches could be promising tools to reduce the caffeine content. For instance, the expression of the *caffeine synthase* (CS) gene was suppressed by RNAi technology. This method has been successful in reducing caffeine content by 44-61 % and theobromine content by 46-67 % in transgenic tea plants (87). Other possible approaches are the use of microbial cells and enzymes for bio-decaffeination and *Agrobacterium*-mediated root transformation studies (89). Furthermore, insights into biotechnological methods could offer potential solutions for reducing caffeine content in tea.

Caffeine (1, 3, 7- trimethylxanthine), a purine alkaloid, is one of the renowned metabolites in tea. It is synthesized specifically in tea leaves, not found in roots or stems. The average caffeine content in dry tea leaves is 3 % (90). It influences taste, medicinal and nutritional values, sense of refreshment and stimulates the Central Nervous System (91). The biosynthesis of caffeine in tea involves several key steps (Fig. 4).

A key enzyme specific for caffeine synthesis is *Tea Caffeine Synthase* (*TCS 1*). It exhibits dual functionality with 2 S-adenosyl methionine-dependent N-methyl transferase activities. It also regulates biotic and abiotic stress and

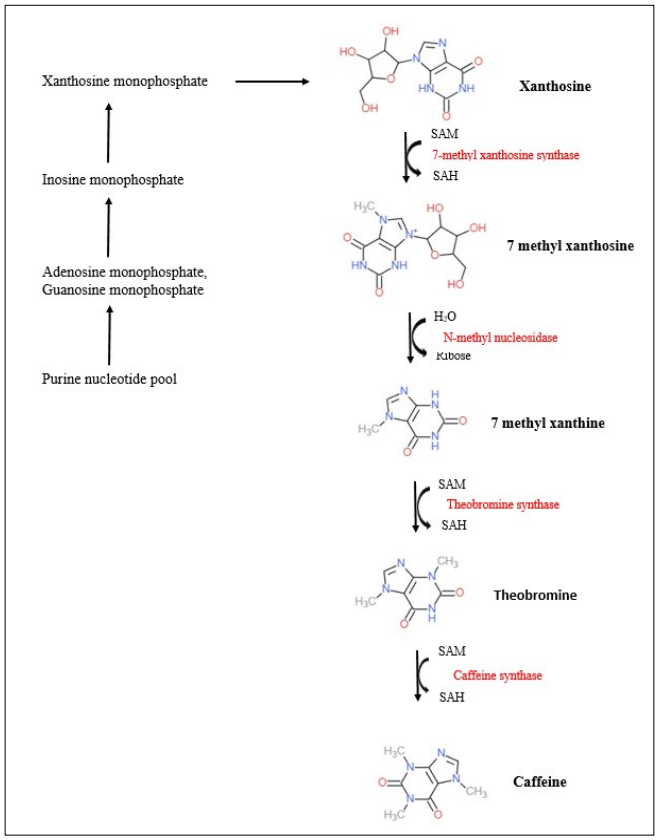

Fig. 4. Caffeine biosynthesis pathway.

SAM: S- Adenosyl Methionine; SAH: S- Adenosyl Homocysteine; H2O: Water

Table 2. Characteristics of *TCS* genes.

Gene	GenBank accession number	Characteristics							
		Size (bp)	Number of exons	Exon start site		Exon stop site	GenBank protein ID	Size of protein	Region of SAM dependent carboxyl methyl transferase
TCS-1	JX647690.1	6916	4	Exon 1	535	627	AFV99128.1	369aa	45- 365
				Exon 2	4489	4884			
				Exon 3	5460	5732			
				Exon 4	6554	6901			
TCS-2	JX647691.1	6861	4	Exon 1	472	549	AFV99129.1	365aa	58- 361
				Exon 2	3855	4253			
				Exon 3	4829	5101			
				Exon 4	5913	6260			
TCS-3 pseudo gene	JX647692.1	5087	-	-	-	-	-	-	-
TCS-4	JX647693.1	7344	4	Exon 1	588	662	AFZ93516.1	365aa	57- 361
				Exon 2	3672	4073			
				Exon 3	4659	4931			
				Exon 4	5675	6022			
TCS-5	JX647694.1	6636	4	Exon 1	546	620	AFZ93517.1	365aa	39- 361
				Exon 2	1810	2211			
				Exon 3	2801	3073			
				Exon 4	4985	5332			
TCS-6 pseudo gene	JX647695.1	5719	-		-	-	-	-	-

contributes to plant development. The family of *TCS* gene comprises 6 genes namely, *TCS2, TCS3, TCS4, TCS5* and *TCS6*. (Table 2) explains about brief overview of the characteristics of these genes. Among these, *TCS1* is most prominently associated with caffeine biosynthesis, showing the highest expression level in the leaves (92). It consists of 1438 bp and encodes 369 amino acids. Purification studies have revealed that caffeine synthase is a monomeric enzyme with a molecular weight of 41 kDa. It exhibits broad substrate specificity, showing highest activity towards 7-methylxanthine, paraxanthine and theobromine. Whereas no activity was reported towards 3- methylxanthine and 1-methylxanthine. The transcription activators of the *TCS1* gene belong to the *MYB* family, with *CsMYB184, CsMYB85* and *CsMYB86* having key roles in biosynthesis (93).

Transcriptomic studies revealed that 132 transcription factors from 30 families are associated with the expression of *TCS1* (94). Higher transcript levels of *Tea Caffeine Synthase* (*TCS1*) have been reported in developing young leaves compared to fully developed leaves, which reflects their distribution level in the leaves. *TCS1* is expressed in the palisade tissues of leaves, where it is believed to participate in the biosynthesis of caffeine (95). Synthesized caffeine accumulates in vacuoles of the cell, where it binds to chlorogenic acid. Notably, expression levels in detached leaves were stable irrespective of various environmental stresses such as exposure to cadmium chloride or different nitrogen sources for two hours. However, during drought stress, a significant decrease in *TCS1* expression is observed, with levels recovering after rehydration. The six allelic variations of the *TCS 1* gene include *TCS1a, TCS1b, TCS1c, TCS1d, TCS1e* and *TCS1f*. Among these, *TCS1a* and *TCS1f* are the predominant and rare alleles found among tea and other related species respectively. Allelic variation of *TCS1* has a crucial role in the final steps of caffeine biosynthesis. A reduction in caffeine biosynthesis may occur due to either low transcription levels of *TCS1* alleles or if the encoded protein exhibits only TS activity (70).

Our previous research work involved the standardization of callus induction and shoot initiation from *ex vivo* grown tea plants and producing decaffeinated tea from callus tissues (96). Currently, we are focussed on developing an efficient, rapid regeneration protocol which paves the way for successful gene transformation studies thereby improving the tea products with value-added traits. Emphasis on decaffeinated tea is gaining immense popularity because of its several health benefits. Thus, the development of an efficient genetic transformation protocol for producing decaffeinated tea will definitely be a groundbreaking discovery in the tea breeding program.

10. Way forward in Gene transformation studies

CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated) genome editing has revolutionized modern biology by enabling site-specific editing of genes that induce selective mutations. This 2-component system is characterized by Cas9 nuclease and guide RNA. Single guide RNA (sgRNA) is a combination of variable crRNA (CRISPR RNA) and constant tracrRNA (trans-activating CRISPR RNA). Designing an efficient sgRNA is critical for maximizing gene editing efficiency. The tracrRNA provides a scaffold for Cas9 protein binding, while crRNA consists of a 20-nucleotide target sequence that searches for the target gene in the genome. Upon binding, Cas9 induces a single-stranded DNA cleavage, followed by repair through either Homology Dependent Recombination or Non-Homologous End Joining. The type II prokaryotic adaptive immune system, particularly SpCas9 (derived from *Streptococcus pyogenes*), is the most widely used variant of CRISPR/Cas technology. It consisted of 20 nucleotide guide sequences and 5'-NGG-3' PAM (Protospacer Adjacent Motif), where the target sequence should exclude NGG. For optimal editing, the target sequence should have a GC content of 40 %-60 %, minimal off-target effects and limited RNA and DNA bulges. Furthermore, avoiding more than four consecutive T bases and ensuring proper gRNA stem-loop structure and duplex formation is essential for constructing effective sgRNAs (97).

CRISPR/Cas9 was first used to shear the eukaryotic genome. Later, it was applied successfully to cereal crops like rice, wheat, sorghum, tomatoes, etc. More recently, this technology has been applied to ornamental plants, morning glory and woody plants. CRISPR Cas9-based genome editing has also been explored in tea, as depicted in (Fig. 5). Initially, constructed CRISPR Cas9 vector system targeting *TCS1* gene (98). The effect of transcription factor *CsHB1* on caffeine accumulation in tea callus was studied by employing CRISPR/Cas9 mediated gene editing. The result demonstrated that blocking of *CsHB1* significantly downregulated the expression of the *yhNMT1* gene and HPLC results confirmed the reduction of caffeine content in transgenic callus by 97.5 % (99). Additionally, Cas9 expression vectors were constructed for editing of Tea *caffeine synthase* using PCR, overlapping PCR and golden gate cloning technology to target the genome of *Ectropis grisescens*, a tea geometrid pest (100). sgRNA was constructed for *Abd- A*, a *Hox* gene associated with the camouflage nature, specifically inhibiting protein synthesis by targeting the first exon. The study successfully achieved 13 mutations out of 20 attempts, yielding a mutation rate of 65 %, demonstrating the successful application of genome editing in *E. grisescens*.

Looking ahead, tea breeding should be able to encounter the evolving challenges with the overarching aim of

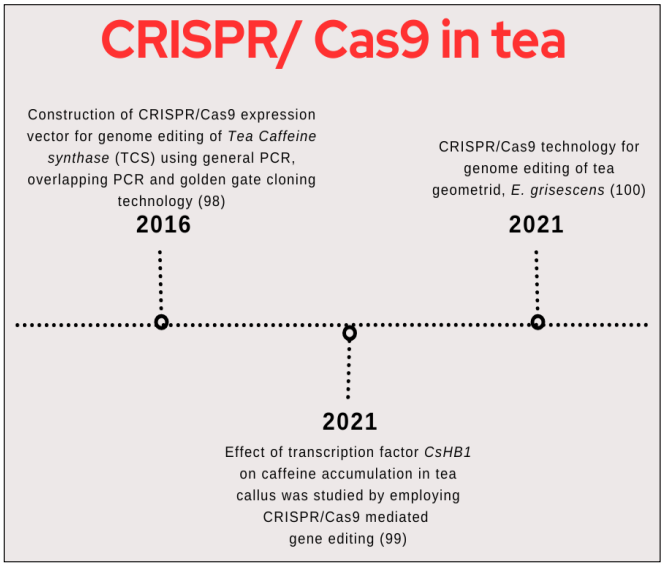

Fig. 5. Historical perspectives of CRISPR- Cas9 mediated transformation in tea.

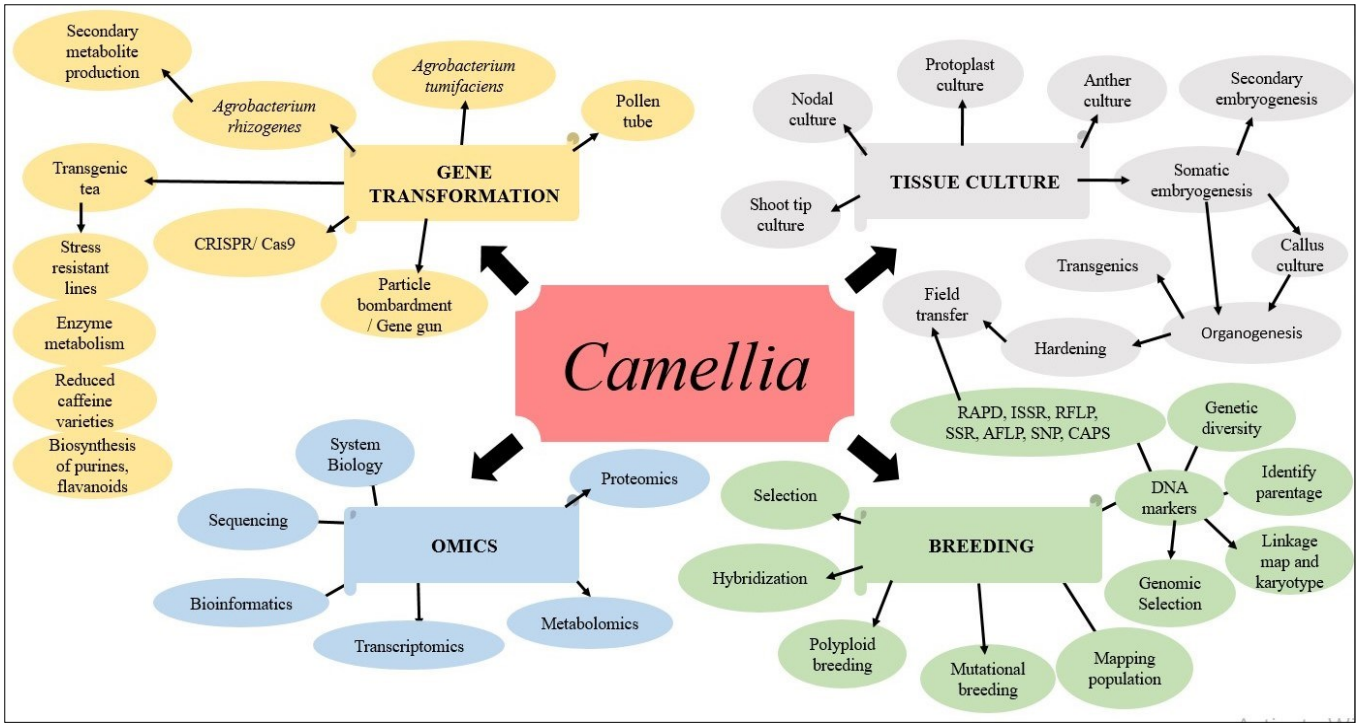

Fig. 6. Convergence of different advanced sciences to improve C. sinensis.

developing improved varieties that address the needs of growers, consumers and the tea industry as a whole while unlocking the opportunities for sustainable cultivation. With a cooperative and collaborative approach across different sectors like agronomy, breeding, genetics, biotechnology, stakeholders and policymakers, it is possible to develop tea crops with improved traits like yield, quality, resistant to pests and diseases, adaptable to changing environmental conditions and ensuring the development of new varieties that aligns with the preference of both producers and consumers (Fig. 6).

Conclusion

Genetic advances in tea are gaining momentum in the current era of research. Although the genetic transformation of tea is a slow process, it has tremendous scope in the near future as there is a need for improved tea lines with enhanced resistance to both biotic and abiotic stress, as well as enriched nutritional and medicinal properties and reduced caffeine content. Efforts are still undertaken by the scientific community for the optimization of effective transformation protocols and standardization of low-cost, highly efficient regeneration systems. These collective efforts will have the potential applications to significantly enhance tea production in this niche area. Thus, the paper presents an extensive analysis of the recent advancements in tea breeding and biotechnological approaches, offering valuable perspectives for future progress.

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Authors' contributions

DKN wrote the first draft of the manuscript, compiled the data and revised the manuscript. NMB conceived the idea, designed the manuscript structure and supplemented the manuscript. VGS, EAAS, NRS and GDK participated in the manuscript preparation and reference organization. All the authors read and approved the final manuscript.

Compliance with ethical standards

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