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Research Paper

Recent Progress of Intra-specific Hybridization of Tea (*Camellia sinensis* (L.) O. Kuntze) in Sri Lanka

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Abstract: Tea germplasm accessions are the most valuable material for tea breeding. However its utilization in the past was limited due to lack of available information. Recently generated information on genetic structure and metabolite profiles of the germplasm were used in selecting parents for hybridization programmes from 2012 to 2016. The main focus of the programme was to make crosses between three taxa of tea *viz*. *Camellia. sinensis* var. *assamica* (Assam type), *C. sinensis* var. *sinensis* (China type) and *C. sinensis spp. lasiocalyx* (Cambod type). Pollinated buds were closely-monitored and the number of crosses, number of fruits, number of seeds and reproductive parameters were recorded. A total of 3978 crosses (2405 direct crosses and 1573 reciprocal crosses) were made and 974 seeds were obtained from 595 fruits. Percentages of fruit set and seed set varied significantly among different families. Irrespective of the inter-subspecies groups, the percentage of seed germination was above 50% and was not significantly-different. Cross compatibility between taxa in terms of percentage of fruit set ranged from 4.6% to 25.6% and varied significantly. Among the inter-subspecies crosses the Assam varieties were found to be the best as female parent. The generated information would be useful for selection of parents and tea improvement programmes in the future.

Keywords: *Camellia sinensis,* Controlled hybridization, Cross pollination, Exotic germplasm, Germination

Introduction

The tea plant [*Camellia sinensis* (L.) O. Kuntze] is a woody-perennial plant of which the tender shoots are used to make the end product. The Asian countries, mainly China, India and Sri Lanka generate more than half of the world tea production. It is an important revenue source for tea producing countries both in terms of earning foreign exchange and generating employment.

The scientific approach to tea cultivation in Sri Lanka began after the establishment of the Tea Research Institute of Ceylon in 1925. The mass selection of vigorous seedlings in the nursery, based on morphological characters, followed by the establishment of seed gardens with these plants by the early planters, marked the beginning of unplanned tea breeding. This approach led to the development of several improved seed populations. This may be considered as the first step towards tea improvement prior to the official release of improved tea cultivars. Initially, introduction of new cultivars from India was followed by selection and large scale multiplication by vegetative cuttings (Gunasekare, 2008).

The final objective of tea breeding is to improve the quality and quantity of the product. High yield, high cup quality, high resistant to biotic and abiotic stresses and, some special characteristics (such as low caffeine, high catechins, etc.) are the main tea breeding objectives in the present stage and predictable future in Sri Lankan tea industry. The scientific community is interested in exploring the health promoting constituents present in tea, namely, flavan-3-ols, flavonols, and their derivatives. Caffeine and polyphenols, the major non-nutrient components in tea, have pharmacological effects. Caffeine, the well-known stimulant, acts on the central nervous system. Scientific investigations have found that polyphenols could act as anti-oxidants after consumption, thus decreasing the risk of many diseases. With the increasing awareness of health benefit of green tea, people have become concerned about functional ingredients, such as methylate catechins, which are beneficial against allergies (Maeda-Yamamoto *et al*., 2001). Abiotic stresses are the principal causes of crop failure and below average yields for most crops. Abiotic stress factors include low temperatures, high salinity, or drought. The amino acid proline accumulates in many plant species in response to environmental stress such as drought, high salinity, high light etc. (Szabados and Savouré, 2010).

Hybridization is one of the main methods of obtaining genetic variation, and it is an important method of breeding new tea cultivars since the parents as well as the hybridized progeny are heterozygous and heterogeneous (Chen *et al*., 2007). Two parents selected for their desired characteristics are crossed to introduce genetic variation. After the initial genetic recombination all subsequent propagation steps are carried out by vegetative propagation. In tea, selection of genotypes is carried out in several steps starting from seed progeny and subsequent selections made from the vegetative propagated plants. Then the performance of these accessions is continuously evaluated 'on station trials' and 'multi locational trials'. Finally accessions with favourable promising results are selected and released as new cultivars (Gunasekare *et al*., 2012).

Tea germplasm is the most valuable and fundamental pre-requisite for tea breeding. Considering the immense genetic diversity of tea germplasm, the future research and development of tea breeding should be focused on integrating biochemical and metabolite profiling approaches into the conventional tea breeding program (Kottawa-Arachchi *et al.*, 2018). Furthermore, the exotic collection of tea germplasm in Sri Lanka includes wild types, cultivated types and Oolong types with unknown pedigree that were conserved in the field gene bank. Most of the accessions in the collection resemble China type and there is a high potential for making use of it for the production of green tea (Kottawa-Arachchi *et al*., 2017). According to the records, only 23 accessions (4% of the total germplasm) have been frequently utilized in tea breeding programmes as parents in the past two decades to develop new cultivars (Gunasekare, 2012). Germplasm activities in the recent past have shifted from collection and conservation efforts towards proper characterization, evaluation and rational utilization of tea germplasm in the current breeding program.

An understanding of morphological (Piyasundara *et al*., 2009), biochemical (Kottawa-Arachchi *et al*., 2013; 2014) and molecular (Mevan *et al*., 2005; Karunarathna *et al*., 2018) diversity among the Sri Lankan tea germplasm accessions is important if the best use is to be extracted in plant improvement programs. Meanwhile, the generated information of recent studies on floral diversity (Ranatunga *et al*., 2017) and metabolite profiling of tea germplasm revealed that results could be effectively used in choosing accessions of desired traits (Punyasiri *et al*., 2017).

All types of tea are made from *C. sinensis*, the major varieties being *C. sinensis* var. *sinensis* (L) and *C. sinensis* var. *assamica* (Masters) recognized as China type and Assam type, respectively. The morphological attributes between the Assam and China varieties being rather sharp and distinct, it was argued that these two varieties should be raised to species status, in addition to recognizing a third variety or southern form, spp. *lasiocalyx*

(Cambod type) as a subspecies of *C. assamica* (Wight, 1962). The current trend is to consider Assam, China and Cambod types as distinct variants or varieties of *C. sinensis* based on their distinctive morphological and morphometric features (Bezbaruah, 1976; Wight, 1962; 1959). Tea is highly heterogeneous, and all the above taxa freely inter-breed, resulting in a cline extending from extreme China types to those of Assam origin. Self-incompatibility and long-term allogamy make the tea plant highly heterogeneous and consequently with broad genetic variation (Chen *et al*., 2005).

The tea germplasm of Sri Lanka is predominantly represented by Cambod-type accessions (68%) followed by Assam types (20%) whereas availability of China type accessions is low. Besides, recent taxonomic studies revealed that the China type accessions in the exotic germplasm will immensely be useful in future tea breeding program as parental lines (Ranatunga *et al*., 2017).

Considering the high genetic diversity of tea germplasm, the future research and development of tea breeding should be focused on integrating biochemical and metabolite profiling approaches into the conventional tea breeding program. Therefore, the present study was focused on conducting controlled hybridization program using inter sub-specific groups as parents for developing diverse progenies for evaluation.

Materials and Methods

Controlled hybridization programs were performed during the period, 2012-2016 using the three *Camellia* sub species *viz. C. sinensis* var. *assamica, C. sinensis* var. *sinensis and, C. sinensis* subsp. *Lasiocalyx* as parents*.* Parental cultivars were selected based on the floral traits (style length, style column length, style arm length, stigma position, petal length, number of petals and sepals) and diversity of metabolites such as catechins, caffeine, anthocyanin and amino acids profile (Punyasiri *et al*. 2017). The selected accessions (Table 1) were maintained throughout the year at appropriate height in tea germplasm.

Table 1. Specific characteristics of parents selected for hybridization

Cultivar	Characteristics
TRI 2043	Introduction, blister blight resistant, high anthocyanin
TRI 3036	TRI developed, high theanine, low proline
TRI 3055	TRI developed, high yield, blister blight susceptible, high caffeine
CV4B1	Estate selection, high theanine and proline
PBGT 41	Exotic, high flavonols, low caffeine, low polyphenols
PBGT 48	Exotic, high flavonols, low polyphenols
PBGT 49	Exotic, high flavonols, low polyphenols
PBGT 61	Exotic, high flavonols, low theanine and proline, low polyphenols
PBGT 68	Exotic, high flavonols, low theanine and proline, low polyphenols

Source: TRI, Sri Lanka, various documents

Hand pollination was conducted from January to April during each year. Pollinated flowers were closely monitored throughout the harvesting period and the number of crosses, number of fruits, and number of seeds were recorded. Harvested seeds were propagated in sand bed and germinated seeds were transferred to nursery bags. All TRI recommended practices were applied to raise healthy plants within the nine-month nursery period. Three reproductive biological parameters; percentage pollination success, percentage reproductive output and percentage of germination success, were also estimated.

Harvested seeds of controlled hybridization program in 2013/2014 were germinated under *in-vitro* condition. Seeds were treated in 20% (v/v) Clorox for surface sterilization and then were washed with sterile distilled water two to three times. Embryos were isolated aseptically from surface sterilized seeds and inoculated *in vitro* on Murashige and Skoog (MS) solid medium supplemented with 3 mg/L BAP, 0.5 mg/L IBA, 8 g/L Agar and 30 g/L sucrose following the previously developed method (Gunasekare and Evans, 2000). The cultures were incubated at 25 °C in 15 h photoperiod. The successfully germinated embryos were sub cultured on to the same medium and multiplied several times at two monthly intervals to increase the number of micro-shoots required for future experiments. Micro-shoots with healthy growth were transferred to the *ex-vitro* rooting medium (Coir dust:topsoil:sand at 1:1:1) and maintained under >80% relative humidity inside the walking type propagator. After two months, rooted micro-shoots were transferred to the nursery bags and maintained at the plant nursery for field planting. Data were analyzed by Analysis of Variance (ANOVA) using the Statistical Analysis Software (SAS version. 9.1).

Results and Discussion

The data on fruit setting and germination during the period from 2012 to 2016 are presented in Table 2. A total of 3978 crosses (2405 direct crosses and 1573 reciprocal crosses) of six families with 12 parental combinations were harvested in the hybridization programme. A total of 974 seeds were obtained from 595 fruits, of which 651 seeds were germinated.

During the hybridization programs from 2012 to 2016, a large number of artificial pollinations of different parental combinations were done. A summary of the pollinations results of various crossing families is presented in Table 3. Generally, certain degree of cross compatibility between taxa has been reported (Bezbaruah and Saikia, 1977). Percentage of fruit set (pollination success) varied significantly among 17 different cross combinations ($P<0.01$; CV = 16.22; 3978 crosses). The maximum percentage of fruit set was shown by the Assam x China followed by Assam x Cambod crosses. Considering the range of percentage fruit-set, direct and reciprocal cross combinations between China and Cambod varieties were found less compatible, whereas Assam x China crosses showed the highest percentage of success. Kumarihami *et al*. (2016) examined the self and cross compatibility among Assam and China types and fruit set was observed only in cross pollination between Assam and China types. Present study revealed that the cross compatibility between Assam and China types was higher than other combinations.

A previous study conducted in India (Bezbaruah and Saikia, 1977), reported that the Assam variety were generally more suitable as pollen sources and less suitable as female parents. But the present study revealed that, the Assam varieties as female parents showed better fruit setting abilities with the China and Cambod varieties present in Sri Lanka. The results revealed the genotypes having variable affinity to different tea varieties*: C. sinensis* var. *assamica*, *C. sinensis* var. *sinensis*, and *C. sinensis* spp. *lasiocalyx* showed dissimilarities in breeding behaviour and in reproductive ability.

Germplasm innovation is a function of pre-breeding. The method of innovation is diverse; a main important genotype innovation is hybridization, including distant hybridization. Distant hybridization is a powerful method for broadening the genetic base of new varieties (Chen *et al*., 2007). Based on morphological and floral traits, the exotic collection is predominantly represented by China type (72%) followed by Cambod type (20%) and Assam type (8%) (Kottawa-Arachchi *et al*., 2017).

The exotic tea germplasm has been used for the first time in hybridization program during 2013 and 1196 crosses were made using three divers exotic accessions as pollen parents whereas TRI 3055 was used as common female parent. Irrespective of the combinations, percentage of fruit set was below 20% in hybrids 2013 and 2014. Among the three combinations, the lowest percentage of fruit set (5.4%) was observed in TRI3055 x PBGT49 followed by TRI3055 x PBGT48 and TRI3055 x PBGT41 crosses. Interestingly, a similar trend was observed with reciprocal crosses in controlled hybridization programme in 2014.

Table 2. Summary of hybridization programs from 2012 to 2016

Hybrid program	Combinations	Taxa	No. of crosses	No. of Fruits	% fruit set (PS)*	No. of Seeds	% seed set (RO)*	No. of seeds per fruit	No. of germinated seeds	% germination
2012	TRI3055 x TRI 2043	Cambod x Assam	200	42	21.0	78	39.0	1.86	66	84.6
2013	TRI3055 x PBGT41	Cambod x Cambod	450	71	15.8	111	24.7	1.56	79	71.2
	TRI3055 x PBGT48	Cambod x China	396	48	12.1	73	18.4	1.52	49	67.1
	TRI3055 x PBGT49	Cambod x China	350	19	5.4	23	6.6	1.21	15	65.2
	TRI2043 x TRI3055	Assam x Cambod	555	124	22.3	196	35.3	1.58	153	78.1
2014 *(In-vitro)*	TRI3055 x PBGT41	Cambod x Cambod	110	10	9.1	13	11.8	1.3	10	76.9
	TRI3055 x PBGT48	Cambod x China	92	5	5.4	5	5.4	1	5	100
	TRI3055 x PBGT49	Cambod x China	100	19	19.0	25	25.0	1.31	17	68.0
	PBGT41 x TRI3055	Cambod x Cambod	300	30	10.0	46	15.3	1.53	38	82.6
	PBGT48 x TRI3055	China x Cambod	226	12	5.3	24	10.6	2	10	41.7
	PBGT49 x TRI3055	China x Cambod	230	9	3.9	16	7.0	1.77	12	75.0
2015	TRI3055 x PBGT41	Cambod x Cambod	132	23	17.4	38	28.8	1.65	18	47.4
	PBGT41 x TRI3055	Cambod x Cambod	262	36	13.7	59	22.5	1.63	42	71.2
2016	TRI3036 x PBGT61	Assam x China	90	23	25.6	45	50.0	1.95	24	53.3
	TRI3036 x PBGT68	Assam x China	280	68	24.3	134	47.9	1.97	73	54.5
	CV4B1 x PBGT61	Assam x China	90	23	25.6	41	45.6	1.78	17	41.5
	CV4B1 x PBGT68	Assam x China	115	33	28.7	47	40.9	1.42	23	48.9

*PS = pollination success; RO = reproductive outputs

<u>Utilization of *in vitro* techniques for conventional breeding program</u>

Aborted or under developed ovaries were found very often in incompatible parents soon after anthesis and intensive abortions of fruitlets were recorded during the initial 15-20 days after pollination despite to the successful pollination (Ariyarathna *et al.*, 2011).

Table 3. Summary of the pollination results of various combinations

Taxa	No. of Crosses	No. of Fruits	% fruit set (PS)*	No. of Seeds	% seed set (RO)*	No. of germinated seeds		% germinated seeds
						NP*	IP*	
China x Cambod	456	21	4.6[c]	40	8.8[c]	-	22	58.4
Assam x Cambod	555	124	22.3[a]	196	35.3[ab]	153	-	78.1
Assam x China	575	147	25.6[a]	267	46.4[a]	137	-	50.0
Cambod x China	938	91	9.7[bc]	126	13.4[c]	64	22	78.1
Cambod x Assam	200	42	21.0[a]	78	39.0[a]	66	-	84.6
Cambod x Cambod	1254	170	13.5[b]	267	21.3[bc]	139	48	69.9

*PS = pollination success; RO = reproductive outputs; NP: nursery propagated, IP: *In vitro* propagated. Means followed by the same letter are not significantly different at P=0.01.

Tea fruit maturation requires 8-9 months after pollination, but abortion of immature fruits was also observed within 5 to 6 months after pollination. Immature embryo rescuing tissue culture strategy is now being developed to improve the success ratio of distant hybridization in the TRI Sri Lanka. The hybridization program in 2014 was focused to generate diverse progeny with the same parents used in previous year as direct crosses and reciprocal crosses. The harvested seeds with viable embryos have been tissue cultured for producing clonal progenies for further evaluations. This has been the first application of successful integration of embryo culture technique in conventional tea breeding program. As a result of *in vitro* propagation of embryos, the germination percentage of several combinations was above 75% (Table 2).

Establishment of hybrid progenies

A progeny was established with one hundred and twenty hybrids generated from hand crossing (both direct and reciprocal crosses) using divers parents TRI3055 and PBGT41 in distant hybridization programs 2013 and 2014. This progeny trial would be a potential source for selecting planting materials with desirable traits such as high quality green and caffeine less tea.

Recently, tea breeders in different tea growing countries have developed anthocyanin-rich purple coloured tea varieties (Joshi *et al*., 2015; Kerio *et al*., 2012). The phenolic compounds such as catechins and caffeine content of various anthocyanin-rich varieties have been compared with green tea leaves. Recent study revealed that the antioxidant values of purple coloured teas were higher than green coloured tea due to the presence of catechins and anthocyanins (Joshi *et al*., 2015). Therefore, tea from anthocyanin-rich cultivars can become specialty teas with high antioxidant activity. Another progeny trial established with one hundred and thirty hybrids generated by crossing two diverse parents, TRI 2043 which is characterized with high pubescence density, pigmented leaves, tolerant to blister blight disease, and TRI 3055 a non-pigmented, high yielding cultivar.

The tea plant is commonly grown in rain-fed ecosystems and thus it encounters seasonal water deficit conditions that induce loss in crop yield. Proline accumulation was significantly higher in the drought-tolerant cultivars in Kenyan germplasm and suggesting that proline concentration could be used as a marker for drought-tolerance in tea (Maritim *et al*., 2015). Therefore, four parental combinations were used to create diverse progeny in hybridization program 2016. Among four germplasm accession used, two cultivars (TRI 3036 and CV4B1) reported high amount of theanine and proline respectively. Other two exotic accessions (CO61 and CO68) recorded low amount of above two amino acids. Another progeny trial has been established using above combination as segregating population for important amino acids *viz.* theanine and proline.

The progenies generated from present controlled hybridization programs can be effectively used in selection of tea accessions with desired traits.

Integrating this information into the conventional tea improvement program to produce new tea cultivars to meet the ever-challenging environment and production of diverse products is vital for future tea breeding program.

Conclusion

Irrespective of the intra-specific, the percentage of seed germination was above 50%. Cross compatibility between taxa in terms of percentage of fruit-set ranges from 4.6% to 25.6% and varied significantly. Among the intra-specific crosses, the Assam varieties were found to be the best as female parent.

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