

Full Length Research Paper

Comparative study on cross-compatibility between *Camellia sinensis* var. *sinensis* (China type) and *C. sinensis* var. *assamica* (Assam type) tea

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Tea has long been a well-known crop for its economic value and widening the genetic variability of tea family is often necessitated. Hybridization programs at intraspecific level have been greatly fascinated as potential and useful methods in tea plant breeding to widening the genetic diversity. This comparative study was intended to explore a new avenue to develop the tea plant breeding programs through evaluating remote intraspecific cross-compatibility between *Camellia sinensis* var. *sinensis* (L.) O. Kuntze and *C. sinensis* var. *assamica* (Masters). Remote intraspecific cross-compatibility was assessed by comparing and contrasting the *in-vivo* pollen germination and pollen tube growth using fluorescence microscopy and the subsequent fruit set following controlled self- and cross-pollinations. *In-vivo* pollen germination and pollen tube growth was examined at 1 day, 3 days, and 14 days after pollination treatments, but disparity was not observed in pollen germination and pollen tube growth between self- and cross-pollinations. Early fruit set was evaluated at 3 months and 6 months after pollination. Fruit set was observed in cross-pollination except self-pollination. A late-acting self-incompatibility system or post-zygotic barriers and close intraspecific cross-compatibility were confirmed within *C. sinensis* var. *sinensis* (L.) O. Kuntze. Potential remote intraspecific cross-compatibility was recorded from cultivars crossed between China type and Assam type tea. The present findings bestow the significant contribution to develop the future tea breeding programs.

Key words: Intraspecific cross, pollen germination, pollen quality, pollination, tea breeding.

INTRODUCTION

Tea has been devoted as the most versatile non-alcoholic beverage in the world. This perennial crop is commercially cultivated for its tender leaves and has been playing

an important role in the world economy. Whilst the Yunnan province in China rewarded as the seedbed of tea plant, currently it has been grown mostly in Southeast

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and South Asian countries (China, Japan, Korea, Sri Lanka, India, and Indonesia), African countries (Kenya, Uganda, and Malawi), and South America as well as up to some extent in North America, Australia, and Europe (Mondal, 2011; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014). The commercially cultivated tea populations belonging to the family Theaceae and the genus *Camellia* have been categorized into three distinct taxa; *Camellia sinensis* var. *sinensis* (L.) O. Kuntze or China type, *Camellia sinensis* var. *assamica* (Masters) or Assam type, and *C. sinensis* var. *assamica* subsp. *lasiocalyx* (Panchon ex Watt.) or Cambod or Southern type (Wachira et al., 2013; Mondal, 2014). The classification of the genus *Camellia* was initially put forwarded by Sealy in 1958 following a revision in 1962 by Wight, principally based on leaf morphological characters of tea plant (Banerjee, 1992; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014). In brief, China type has small size leaves, Assam type has large size leaves, and Cambod or Southern type leaf size is intermediate between of Assam and China types (Banerjee, 1992; Mondal et al., 2004; Chen et al., 2012; Mondal, 2014). Owing to the out-breeding nature of tea plant, the cultivated germplasm consists of extreme China types to extreme Assam types with the continuous variation between them (Banerjee, 1992; Mondal et al., 2004; Rajkumar et al., 2010; Ariyaratna et al., 2011; Chen et al., 2012; Wachira et al., 2013; Mondal, 2014).

Tea has long been a well-known crop for its economic value and widening the genetic variability of tea family is often necessitated. In the recent past, tea plant breeding has been intensified and expanded to widening the genetic variability through accelerating the production of new improved plant materials. Hybridization programs at intraspecific level have been greatly fascinated as potential and useful methods in tea plant breeding to widening the genetic diversity. The existing tea populations all over the world might be as a result of the intensive natural hybridization between three main taxa and other non-tea *Camellia* species (Bezbaruah and Gogoi, 1972; Banerjee, 1992; Mondal et al., 2004; Rajkumar et al., 2010; Ariyaratna et al., 2011). To the best of our knowledge attempts on remote intraspecific hybridizations between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* have not been reported even though close intraspecific hybridization within each China or Assam type has been undertaken extensively in *C. sinensis*. Novel tea cultivars with blended desirable traits such as biotic and abiotic stress resistance, new aroma of tea, and specific characters in chemical components might be accomplished fruitfully via remote intraspecific hybridization between China and Assam types.

Thus, this comparative study is intended to explore a new avenue to develop the tea plant breeding programs through evaluating remote intraspecific cross-compatibility between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* by comparing and contrasting the *in-vivo* pollen

germination and pollen tube growth using fluorescence microscopy and fruit set following controlled self- and cross-pollinations.

MATERIALS AND METHODS

Plant

Present experiment used three cultivars of *C. sinensis* var. *sinensis* (L.) O. Kunze namely, 'Yabukita', 'Yutakamidori', 'Okuhikari' and one cultivar of *C. sinensis* var. *assamica* (Masters) namely, 'AI-37'. The tea plants were grown in plastic greenhouse located at Jeju National University, South Korea. *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' were used as pollen donors for artificial pollination. *C. sinensis* var. *sinensis* 'Yabukita' is a mid-plucked and leading tea cultivar in Japan, cultivated in about 75% of country's tea fields. It has been proved to be a good yielding, cold resistant, and good seed setting cultivar with intense green tea flavor (Ogino et al., 2009; Yagi et al., 2010; Chen et al., 2012). *C. sinensis* var. *sinensis* 'Yutakamidori' is an early-plucked and second most cultivated tea cultivar in Japan and has good yield as well as highly resistant to anthracnose (Yagi et al., 2010). *C. sinensis* var. *sinensis* 'Okuhikari' is late-plucked and resistant to anthracnose, blister blight, and gray blight but susceptible to bacterial shoot blight. This cultivar has yield potential similar to *C. sinensis* var. *sinensis* 'Yabukita' (Yagi et al., 2010). Assam type tea cultivars have been renowned as potential genetic resources in tea plant breeding due to their disease resistant traits and fine tea aroma (Takeda, 1990).

Pollen collection

Late balloon phenophase flower buds at pre-anthesis stage were collected for pollen collection. Anthers were collected using fine forceps onto tracing papers laid in petri dishes and air dried at room temperature (20 to 25°C) for 2 to 3 days until anthers were dehiscent and released pollen grains. Collected pollens were stored at -4°C in glass tubular vial bottles with air-tight caps until used for pollination.

Pollen quality tests

Collected and stored pollens were initially subjected to pollen quality tests viz. viability and *in-vitro* germinability using staining method and *in-vitro* germination method, respectively.

Pollen viability was determined with two different staining tests, that is, fluorescein diacetate-FDA test (Heslop-Harrison and Heslop-Harrison, 1970) and iodine potassium iodide-I₂KI test. The pollen grains were separately immersed in a trace of 1% I₂KI solution and FDA solution (200 µg·mL⁻¹ FDA in 0.5 M sucrose) in eppendorf tubes following 5 min incubation at room temperature in dark condition for proper staining of pollen grains. A drop of stained pollen mixture was mounted on a glass slide and covered with a coverslip as the drop of pollen evenly distributed. Pollen viability counts were made under the light microscope and the fluorescence microscope for I₂KI and FDA tests, respectively. Six microscopic slides were used for each cultivar in each staining method. In relation to I₂KI test, pollen grains stained with dark brown in color were counted as viable while yellowish or unstained pollen were counted as non-viable (Figure 1A). In contrast, in FDA test the viable pollen grains fluoresced brightly and non-viable pollen emitted the ghost fluorescence (Figure 1B). The percentage of pollen viability was determined as ratio of the number of viable grains to the total number of grains per viewed area.

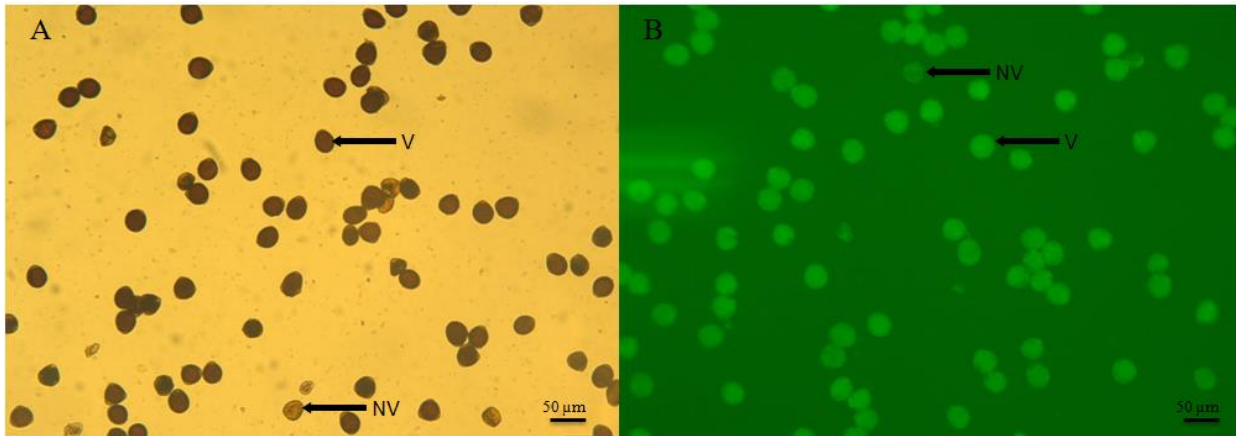


Figure 1. Viable and non-viable pollens in I₂KI (A) and FDA (B) staining tests. V: viable; NV: non-viable.

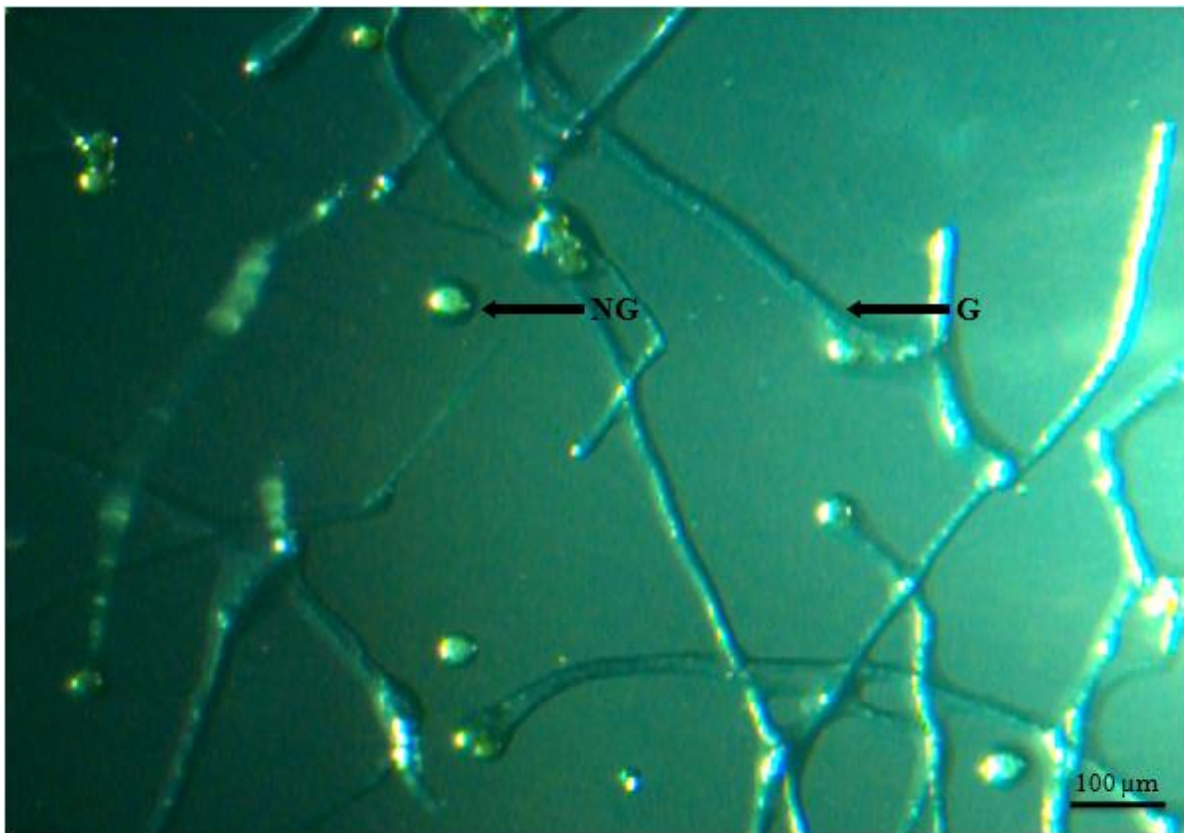


Figure 2. Germinated and non-germinated pollen grains in *in-vitro* germination medium. G: germinated; NG: non-germinated.

In-vitro pollen germination was assessed via “Hanging Drop” method described by Yang et al. (2008). Pollen were uniformly scattered on to the media consisted with 1% agar and 10% sucrose with pH 5.6 in petri plates. Pollen germination was observed under the light microscope after 4 h incubation period in dark. Pollen grains were considered as germinated when the pollen tube length was equal to or greater than the diameter of pollen grain (Figure 2). Five plates were made for each cultivar. The percentage of pollen

germination was calculated as ratio of the number of germinated grains to the total number of grains per viewed area.

Pollination treatments

Artificial pollination was carried out in plastic green house in October, 2014 when the peak flowering occurred. Late balloon

Table 1. The percentages of pollen viability and *in-vitro* germination of *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'Al-37'.

Cultivar	Viability (%)		<i>In-vitro</i> germination (%)
	I ₂ KI	FDA	
'Yabukita'	88.5±1.5 ^z	82.2±1.0	69.43±0.9
'Al-37'	87.5±1.2	81.3±1.1	81.05±1.3

^zValues indicate means ± S.E.

phenophase flower buds of ovule parents at pre-anthesis stage were emasculated by removing petals and stamens using fine forceps and hand pollinated with the aid of a small camel's hair brush and then bagged. Pollination treatments were as follows: 'Yabukita' x 'Yabukita' self pollination and 'Yutakamidori' x 'Yabukita' close intraspecific cross pollination within *C. sinensis* var. *sinensis*, and 'Yabukita' x 'Al-37' and 'Okuhikari' x 'Al-37' remote intraspecific cross pollinations between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. Fifty flower buds for each combination were used.

***In-vivo* pollen germination and pollen tube growth**

In-vivo pollen germination test was done using aniline blue fluorescence microscopy assay following Yang et al. (2008). Self- and cross-pollinated flower pistils were collected at 1 day, 3 days, and 14 days after pollination and fixed in FAA (70% ethanol: formalin: acetic acid, 18: 1: 1, v/v/v). Five pistils from each treatment were collected. Fixed pistils were rinsed with distilled water for 4-5 times. Cleared pistils were hydrolyzed in 2 N NaOH at 60 °C for 1 h until the tissue became transparent. Hydrolyzed pistils were rinsed in distilled water for 4-5 times and stained with 0.1% aniline blue dissolved in 0.1 N K₃PO₄ for 24 h at room temperature in dark place. The stained pistil was placed on a microscopic slide and squashed under a glass coverslip to spread the material evenly and observed under the fluorescence microscope (Leica DMREB, Leica Co., CA, US).

Early fruit set

Some of pollinated flowers were left on plant to monitor the early fruit set for self- and cross-pollinations which were recorded at 3 months and 6 months after pollination. The percentages of fruit set and fruit diameters were recorded.

RESULTS

Pollen quality tests

The data on pollen quality tests viz. viability and *in-vitro* germinability were presented in Table 1. In I₂KI test, *C. sinensis* var. *sinensis* 'Yabukita' had the highest (88.5%) pollen viability followed by *C. sinensis* var. *assamica* 'Al-37' (87.5%). Pollen viability determined by FDA test was 82.2% for *C. sinensis* var. *sinensis* 'Yabukita' and 81.3% for *C. sinensis* var. *assamica* 'Al-37'. The highest *in-vitro* pollen germination was obtained for *C. sinensis* var. *assamica* 'Al-37' (81.05%) and it was significantly low in *C. sinensis* var. *sinensis* 'Yabukita' (69.43%).

***In-vivo* pollen germination and pollen tube growth**

In the present study, the *in-vivo* pollen germination and pollen tube growth related to the controlled self- and cross-pollinations were examined at 1 day, 3 days, and 14 days after pollination using fluorescence microscopy. A specialized polysaccharide viz. "callose" found in pollen tube wall has a great affinity to aniline blue and produces a bright yellow-green fluorescence when illuminated by ultraviolet light. The growing pollen tubes are characterized by callose outlining and irregularly spaced callose plugs in pollen tubes (Kho and Baer, 1968; Unal et al., 2013). This phenomenon was used to detect pollen germination and pollen tube growth in pistils using fluorescence microscopy. Four broad sites viz. stigma, upper style, lower style, and ovary in pistil were examined for pollen germination and pollen tube growth. The style of tea flower pistils consists of three arms which are united for varying length into a column (Banerjee, 1992; Mondal et al., 2004; Ariyaratna et al., 2011). Therefore, most of pollen tubes were overlapped with each other subsequent to squashing of pistils. Consequently, the quantification of precise number of pollen tubes at each site of pistils was not feasible in our study.

Fluorescent microscopy revealed that the copious pollen grains had successfully germinated on stigma and grew rapidly through style in a dense cluster within 1 day after pollination in both self- and cross-pollination (Figure 3). Within one day of pollination the elongated pollen tubes were found in upper and lower style and tails of pollen tubes were observed in ovary of both selfed and crossed flower pistils (Figure 3). The squashed selfed and crossed pistil samples were not clear enough to examine the pollen tubes in or near ovules. The magnitude of the pollen tubes germinated on stigma was higher than that of reaching to style base and to ovary in all tested crosses. The present study further revealed that at 3 days and 14 days after pollination there were less pollen grains and pollen tubes in all pistils than those found at 1 day after pollination. Moreover, as the time after pollination prolonged, the fluorescence of pollen tubes disappeared and recording the presence of pollen tubes based on callose deposition was not feasible with 3 days and 14 days pistil samples. Even so, the obvious morphological or structural dissimilarities in pollen grain germination and pollen tube growth were not found in self- and cross-

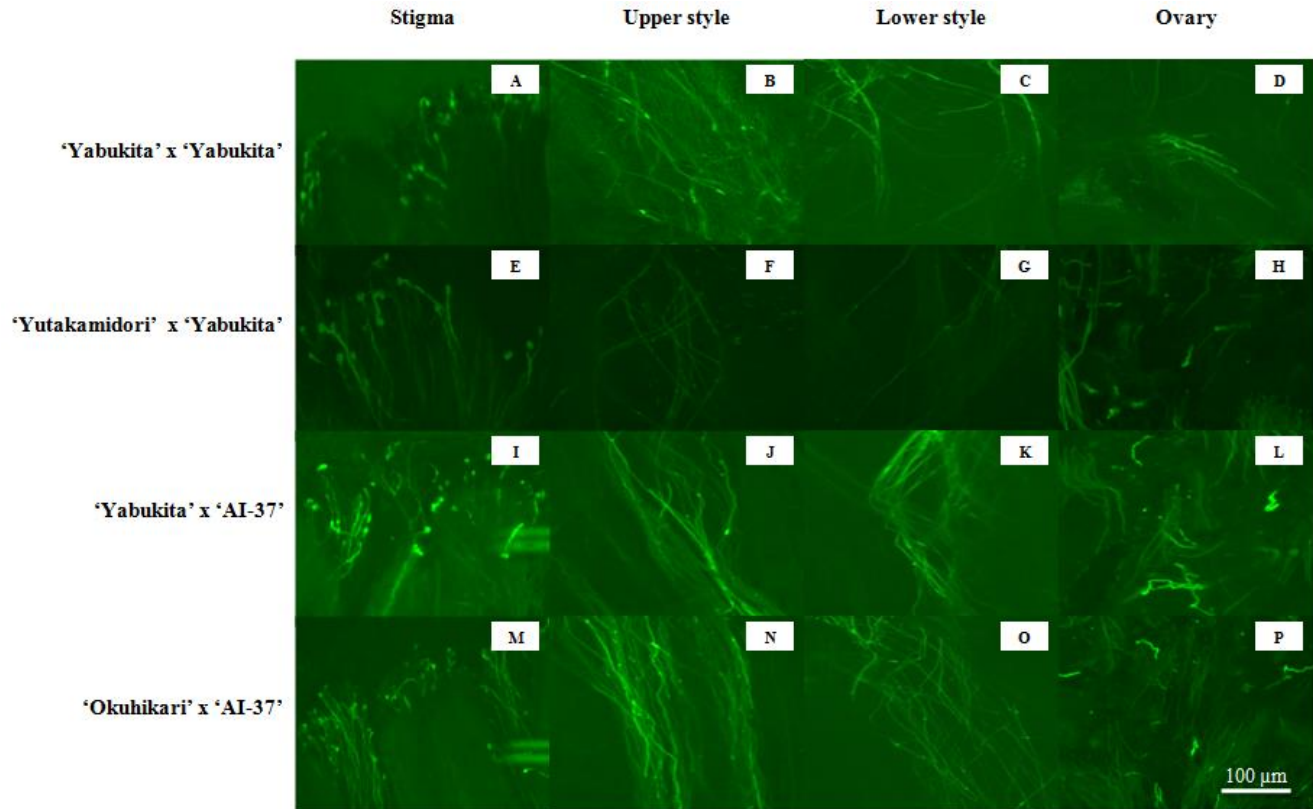


Figure 3. *In-vivo* pollen germination and pollen tube growth from stigma to ovary visualized using fluorescence microscopy at one day after different pollination treatments. (A–D) ‘Yabukita’ x ‘Yabukita’; (E–H) ‘Yutakamidori’ x ‘Yabukita’; (I–L) ‘Yabukita’ x ‘AI-37’ and (M–P) ‘Okuhikari’ x ‘AI-37’.

combinations. Pollen tube growth was normal without any considerable inhibition of pollen germination and pollen tube growth in pistils and showed same pattern for all type of crosses in present investigation. Disregard to the normal growth pattern an unusual zigzag growth was discriminated at very low frequency in self-cross (‘Yabukita’ x ‘Yabukita’) and remote intraspecific crosses (‘Yabukita’ x ‘AI-37’ and ‘Okuhikari’ x ‘AI-37’) (Figure 4).

Early fruit set

Early fruit set percentages and fruit diameters were recorded at 3 months and 6 months after pollination (Table 2). The estimated fruit set percentages at 3 months after pollination were, 75%, 60%, and 80% for crosses ‘Yutakamidori’ x ‘Yabukita’, ‘Yabukita’ x ‘AI-37’ and ‘Okuhikari’ x ‘AI-37’, respectively. The fruit set percentages declined at 6 months after pollination as 70%, 35%, and 50% for above crosses, respectively. Fruit diameters ranged between 4.4 to 4.8 mm and 4.5 to 4.9 mm at 3 months and 6 months after pollination, respectively. In self-cross ‘Yabukita’ x ‘Yabukita’ most of pistils were withered and dropped within a few days after pollination.

DISCUSSION

Pollen quality tests

Microscopic pollen grain contains the male gamete to be used in fertilization. Assessing the pollen quality for a cultivar to be used as a pollinizer is essential in plant breeding to ensure the success of artificial pollination. Pollen quality of *C. sinensis* var. *sinensis* ‘Yabukita’ and *C. sinensis* var. *assamica* ‘AI-37’ was evaluated before using them in controlled pollination. Heslop-Harrison et al. (1984) perceptively reviewed three general approaches for evaluating pollen quality viz., histochemical, *in-vitro* and *in-vivo* pollen germination and pollen tube growth. These tests estimated the potential of pollen to germinate and grow on stigma in artificial pollination. Histochemical tests are based either on the ability to stain specific constituents of vegetative cell of pollen grain or on the activity of specific enzymes (Heslop-Harrison et al., 1984).

In the present study, I₂KI and FDA histochemical tests were used for pollen viability assessment. A significant difference was not found between *C. sinensis* var. *sinensis* ‘Yabukita’ and *C. sinensis* var. *assamica* ‘AI-37’ for each of viability tests. Pollen viability detected by I₂KI

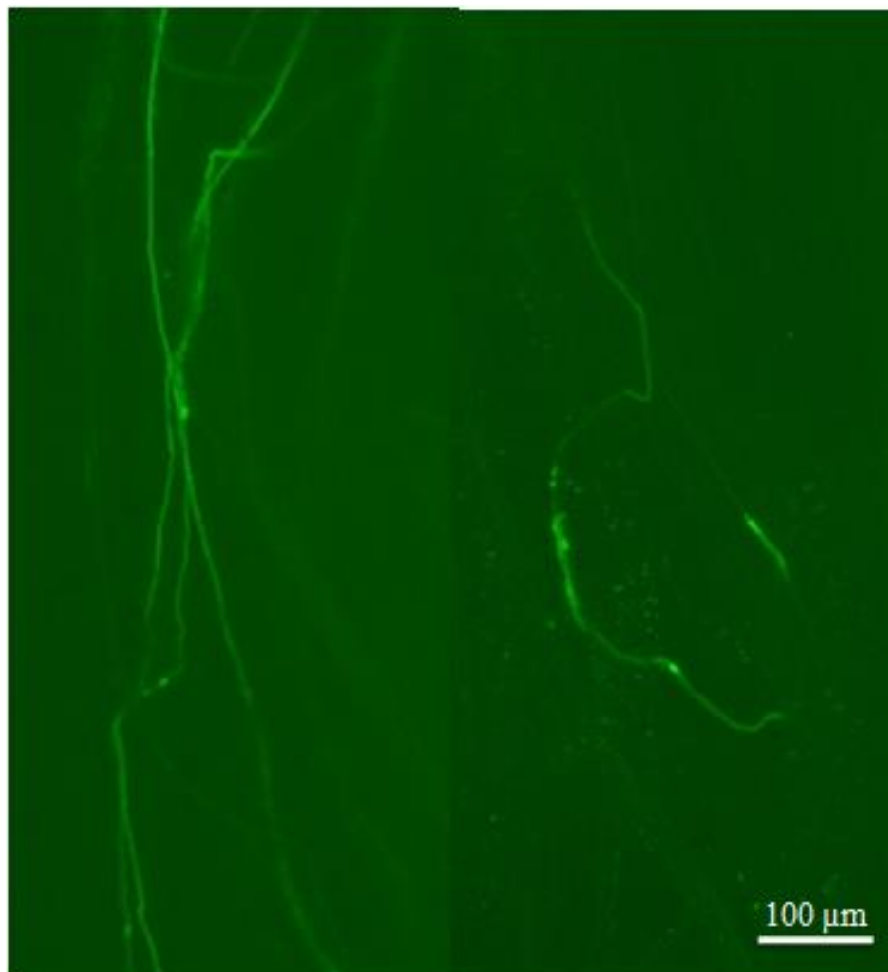


Figure 4. Abnormal 'zigzag' growth pattern observed at 3 days after pollination in lower styles at very low frequency in self cross 'Yabukita' x 'Yabukita' and remote intraspecific crosses 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37'.

Table 2. The percentages of fruit set and fruit diameters in cross-pollination.

Cross	Fruit set (%)		Fruit diameter (mm)	
	3 Months AP	6 Months AP	3 Months AP	6 Months AP
'Yutakamidori' x 'Yabukita'	75	70	4.4	4.5
'Yabukita' x 'AI-37'	60	35	4.7	5.0
'Okuhikari' x 'AI-37'	80	50	4.8	4.9

AP: After pollination.

test has given higher values for each cultivar than by FDA (Table 1). The I₂KI indicates the presence of starch while FDA implies the integrity of plasmalemma of vegetative cell of pollen grains (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984). Hence, FDA test was more effective in tea pollen viability assessing than I₂KI test.

In *in-vitro* pollen germination and pollen tube growth

test involves germinating pollen on artificial media and determining the germinability and pollen tube growth (Heslop-Harrison et al., 1984). The percentage of *in-vitro* pollen germination of two pollen parents *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' was found to be significantly different (Table 1). The percentage of *in-vitro* pollen germination is low in both cultivars when compared to pollen viability percentages.

This clearly indicated that all the pollen estimated by staining methods to be viable was not germinated in *in-vitro* medium. Hence, compared to *in-vitro* germination test the pollen staining tests overestimated the viability of pollen. *In-vitro* pollen germination is generally believed to provide more reliable estimate of pollen viability (Muoki et al., 2007). Additionally, both viability and *in-vitro* germinability tests together provided important insight into understanding about the pollen quality. Notably, disparities in pollen quality are evidenced for potential male gametophyte competition and unequal reproductive success among *C. sinensis* genotypes (Muoki et al., 2007). The paternal traits, that is, phenology of male organ and amount of pollen produced, and pollen grain traits, that is, germination percentage, germination time, pollen tube growth rate, and selective fertilization are the factors that determine the fitness of pollinizers (Muoki et al., 2007).

In this study, paternal parents *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'Al-37' possessed considerable good quality in both viability and *in-vitro* germinability tests which is a prerequisite for successful pollination and fertilization. Therefore, both of tea cultivars, *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'Al-37' can be considered as good pollinators.

***In-vivo* pollen germination and pollen tube growth**

Monitoring the pollen grain germination and pollen tube growth in pistils using aniline blue fluorescence microscopy assay subsequently with the fruit and seed set are included in cross-compatibility test in intraspecific hybridization (Heslop-Harrison et al., 1984; Muoki et al., 2007). Although fluorescence microscopy is a practicable approach for examining the pollen tube growth in pistil, this method is relatively time consuming, unfeasible for testing many samples. Further, seed set may depend not only on fertilization, but also on post-pollination development of ovary, pistil receptivity, and incompatibility reactions (Heslop-Harrison et al., 1984).

In our study, successful germination of pollen grains on stigma, rapid growth of pollen tubes through style, and tails of pollen tubes in ovary were examined within one day after pollination of both selfed and crossed flower pistils. This was supported by further evidenced from Wachira and Kamunya (2005) with an account of tea pollen germination on stigma and the succeeding pollen tube growth along style of self- well as cross-pollination within one day. In addition, Simura and Oosone (1956) monitored satisfactory pollen grain germination on stigma within about 1 h after both cross- and self-pollination of *C. sinensis*. The successful pollen germination on stigma in all cross combinations indicated the pistils' receptivity during the pollination. Stigmatic receptivity showed the ability of stigma to support the pollen germination. The selected late balloon phenophase flower buds of ovule

parents in this study proved to being well receptive at the time of pollination. This fact was further emphasized by Ariyaratna et al. (2011), by examining the adequate pollen adhesion and germination in manually pollinated floral buds at balloon stage. Tea flowers own a group III wet type stigma (Heslop-Harrison and Shivanna, 1977; Ariyaratna et al., 2011) and stigma surface is the first site of the cross compatibility and incompatibility responses that govern the success of the breeding system (Heslop-Harrison and Heslop-Harrison, 1985). Further, this implies the affinity of plant materials used in hybridization program. In view of the self-pollen grains germinated on stigma in our study it is convinced the gametophytic self-incompatibility of tea plant (Fuchinoue, 1979; Chen et al., 2012). The occurrence of pollen tubes in ovary might be applied as a reliable appraisal to persuade the ovule penetration in self-/cross-pollinated pistils. Our results are compatible with the findings of Chen et al. (2012) who observed the successful pollen tubes elongation through style to ovary at 24-48 h after self-/cross-pollination in *C. sinensis*. Supporting to our observations, Rogers (1975) also reported that by 24 h crossed/selfed pollen tubes had entered ovary and probably penetrated as far as the ovules. Analogous growth pattern of cross- as well as self-pollen tubes in tea plant flowers has been reported by Wachira and Kamunya (2005).

Higher magnitude of pollen tubes was also observed to have germinated on stigma than that of reaching to style base and to ovary in all tested crosses. Further, at 3 days and 14 days after pollination the fluorescence of pollen tubes disappeared and recording the presence of pollen tubes based on callose deposition was not feasible. These observations might have resulted from the degradation of growth substances in pollen grains and pistils. According to the information generated by Rosen (1971), the pollen germination and pollen tube growth on stigma rely on reserves within the pollen, hence, this phenomenon is known as autotrophic. The pollen tube growth in styles is heterotrophic, since, the growing pollen tube depends on stylar reserves. Thus, the growing pollen tubes might be competing for nutrients and space during their autotrophic and heterotrophic growth in pistil and number of pollen tubes gradually decreased from stigma to ovary.

Seeing as the pistil sampling was not done before one day after pollination and pollen tubes grew along style and reach to ovary within one day the speed of pollen tube elongation was not distinguishable between self- and cross-pollinations in current investigation. Nevertheless, the review of literature on this subject reported by many scholars helped to have a clear understanding of our observations comparatively. Chen et al. (2012) have determined the pollen tube elongation rate in *C. sinensis* with the ration of length of the longest pollen tube to that of the style and differences was found based on cultivars. It was found that the higher elongation rate

for cross-pollination and lower rates for self-pollination in some cultivars while there was not a substantial difference in pollen tube elongation rate between cross- and self-pollination in some other cultivars (Chen et al., 2012). Liao et al. (2014) also supportively depicted that the growth speed of crossed pollen tubes of *C. oleifera* was slightly faster than selfed pollen tubes as pollen tubes reached style base at 48 h after cross-pollination and 60 h after self-pollination. Comparable remarks were reported by Simura and Oosone (1956) where, in crossed flowers the pollen tubes grew rapidly and reached funiculus base in about 36 to 40 h after pollination, while in selfed flowers they grew uniformly and scarcely reached it by 72 h. Moreover, pollen tubes grew slower in styles of a different species and protruded ovules within 3 to 5 days after pollination (interspecific crosses) whereas that of within the same species (intraspecific crosses) occurs within 1-2 days after pollination (Hwang et al., 1992). Wachira and Kamunya (2005) found that the cross- and self-pollen tubes of tea plant flowers grow at different rates and compete to fertilize the ovule.

The pollen grain germination and pollen tube growth pattern was normal and similar in self- and cross-combinations in our study. Tanaka (1988) and Liao et al. (2014) reinforced our appraisal by particular studies on self-incompatibility in the genus *Camellia*. Aside from the normal growth of pollen tubes we observed an unusual zigzag growth at very low frequency in self and remote intraspecific crosses. Conspicuously, Hwang et al. (1992) has been reported a small frequency of abnormal pollen tubes with a zigzag or branching growth habit in interspecific crosses between *C. japonica* and *C. chrysantha*. Apart from that the distorted pollen tubes containing reversal tubes, swelling tube tips with callose deposits, irregular tubes and furcal tubes have been noted in selfing pistils of *C. oleifera*, an another member of the genus *Camellia* (Liao et al., 2014). Another report by Rogers (1975) has been documented the presence of self-pollen tubes with swollen and distorted tips in some tea clones.

Early fruit set

So as to confirm remote intraspecific cross-compatibility of crosses attained in this experiment, *in-vivo* pollen tube growth is not a sufficient witness since, pollen tube growth patterns in selfed and crossed pollinated pistils were similar. In view of that, the cross- as well as self-pollen tubes had reached ovary and might be near the ovules at 24 h after pollination. Therefore, it was intended to go into an estimation of fruit set and retention subsequent to the self-and cross-pollination.

Early fruit set and retention of all cross combinations was estimated at 3 months and 6 months after pollination. In our observations the cross-pollinations bore fruits whereas self-pollination failed in fruiting. The fruit set percentages declined at 6 months after pollination and

obvious reductions were recored for the remote intraspecific cross pollinations, 'Yabukita' x 'AI-37' and 'Okuhikari' x 'AI-37' compared to the close intraspecific cross pollination, 'Yutakamidori' x 'Yabukita'. Aborted or under developed ovaries were found very often in tea bushes soon after anthesis and intensive abortion of fruitlets/seeds were recorded during the initial 15 to 20 days after pollination despite to the succeeded pollination (Ariyaratna et al., 2011). Hence, comparatively obvious decline in fruit set percentages in remote intraspecific cross pollinations might be caused by the high intensive abortion of fruitlets/seeds. In self-pollination most of pistils were withered and dropped within a few days after pollination. According to the depiction by Ariyaratna et al. (2011) in incompatible cross combinations more than 90% of pollinated flowers withered and fell in less than one week. Similar circumstance has been documented by Ozaki et al. (2003), that is, unfertilized fruitlets dropped before one month after pollination in the genus *Camellia*. With an interest Ozaki et al. (2003) further elucidated exceptional fruit set on few self-pollinated cultivars of *C. japonica* L. which showed 5-27% fruit set with scarce of perfect seeds. A report by Simura and Oosone (1956) mentioned fruiting rates of tea plant as 20 to 30% in crossed flowers and 3 to 10% in selfed flowers and time taken for double fertilization after pollination is 36-48 h and 62-72 h in crossed and selfed flowers, respectively. Tea fruit maturation requires 8 to 9 months after pollination and possesses two seeds/fruit on average with maximum of six seeds/fruit depending on parents (Ariyaratna et al., 2011). Indirectly, based on percentage of fruit set we can have a clear idea on percentage of pollination success of each cross combinations as described by Ariyaratna et al. (2011). Percentage of pollination success was defined as the percentage of fruit set per each of pollinated flower (Ariyaratna et al., 2011).

Thus, the merger of pollen tube growth observations with fruit set after self- and cross-pollination is most useful to determine the self-/cross-compatibility. In the present observations the pollen tube could be reached to ovary in self- and cross-pollinated cultivars. Regardless of *in-vivo* pollen tube growth the fruiting was conflicted between selfed and crossed pollinations. As selfed cross 'Yabukita' x 'Yabukita' failed in fruit set it proved that the fertilization was not occurred in that particular cross. Conversely, the cross pollinations eventually developed fruits owing to unbeaten fertilization due to successful pollen tube penetration into ovules. Therefore, we can presume that the self-pollen tubes of *C. sinensis* might have not entered ovules or they might have failed in fertilization after entered ovule. Thus, there might be post-zygotic barriers to overcome selfing rather than pre-zygotic barriers. Hence, the contemporary results further confirmed the late-acting self-incompatibility present in *C. sinensis*. Self-incompatibility of tea plant has been adequately appraised by numerous studies over the past decades. More recently, self-incompatibility in tea plant

has been comprehensively explored by Wachira and Kamunya (2005) and Chen et al. (2012) with aniline blue fluorescence assay and sturdily confirmed the self-incompatibility of tea plant as a late-acting self-incompatibility system or an ovarian sterility. This heritable reproductive phenomenon of tea plant has been further reviewed by Rogers (1975) and Fuchinoue (1979). Self-incompatibility of *Camellia* spp. contributes to huge genetic variation within the genus. The close intraspecific cross within *C. sinensis* var. *sinensis*, 'Yutakamidori' x 'Yabukita' showed positive responses in all examined criteria as discussed earlier and it confirmed out-crossing nature of tea plant. The close intraspecific hybridization in *C. sinensis* is extensively utilized and several hundred cultivars have been resulted from this hybridization technique in all tea growing countries (Takeda, 1990; Chen et al., 2012).

Last but not least, the successful pollen germination and pollen tube growth in pistils as far as to ovary of remote intraspecific cross combinations between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica* with considerable fruit set did not indicate any obvious pre-zygotic barrier(s). The post-zygotic barriers have not been studied yet in the present intraspecific hybridization effort. The post-zygotic reproductive barriers, for instance hybrid embryo abortion in the genus *Camellia* have been recognized often in interspecific incompatibility comparing to pre-zygotic barriers (Ackerman, 1971; Hwang et al., 1992). Consequently, by insightful assessing of our research and out comes with previous reports it can be postulated that remote intraspecific hybridization might be feasible between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. Supplementary, the degree of compatibility between these hybridization species showed their genomic affinities to each other.

Conclusion

The present study revealed the effectiveness of remote intraspecific cross-compatibility between *C. sinensis* var. *sinensis* (China type) and *C. sinensis* var. *assamica* (Assam type) by means of *in-vivo* pollen tube growth and subsequent fruit set. This histological approach is known to be a reliable, rapid process to evaluate cross-compatibility of specific crosses. As intraspecific hybridization is highly renowned to breed superior tea cultivars the contemporary findings might be applicable in tea plant breeding programs. Additional experimental trials in several aspects on remote intraspecific hybridization between China type and Assam type tea are mandatory to compose a tangible statement on conclusion of this foremost attempt.

Conflict of Interests

The authors have not declared any conflict of interest.

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