A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Evaluation of Pollen Development and Cross-Compatibility in China and Assam Types of Tea Plants

차나무 중국종과 야생종에서의 화분발달 및 교배친화성 평가

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August, 2015

DEPARTMENT OF HORTICULTURAL SCIENCE
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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(Supervised by Professor **Kwan Jeong Song**, PhD)

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Agriculture

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ABSTRACT

The present study was intended to evaluate the anther and pollen ontogeny and cross-compatibility in China and Assam types of tea plants. Young and mature flowers at different progressive developmental stages were collected. Anther and pollen ontogeny was determined using light microscopy after fixation and staining. Cross-compatibility in China and Assam types of tea plants 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. The results showed tetrasporangiate anthers with a various ontogenetic sequence of pollen (archesporial cells, microspore mother cells, tetrahedral tetrads, uninucleate microspores, and two-celled mature pollen grains). Mature pollen grains are spherical to triangular in shape, tricolporate, and enclosed with the exine and intine. The ontogenetic sequence of anther wall development followed the basic type and composed of an epidermal layer, an endothelial layer, two middle layers, and a glandular type tapetum. Anther dehiscence was taken place by means of longitudinal slits and the mature pollen grains liberated at a bicellular stage. Mature pollen at the late balloon phenophase stage of flower buds possessed considerable good quality by means of viability and germinability which is a prerequisite for successful pollination and fertilization. An analogous anther and pollen ontogenesis in China type and Assam type tea plants was examined while the selected progressive developmental stages of flowers represented a good correlation with the precise stages of anther and pollen ontogeny. 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 of self-pollination and high cross-compatibility of close intraspecific crosses were confirmed in tea plant [*Camellia sinensis* (L.) O. Kuntze]. A potentially similar remote intraspecific cross-compatibility was recorded from the cultivars crossed between China type and Assam type tea. The present findings bestow the significant contribution to develop the future tea breeding programs.

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INTRODUCTION

Taxonomically, tea plant [*Camellia sinensis* (L.) O. Kuntze] has been classified into the family Theaceae and the genus *Camellia*. The tender leaves of tea plant have been used to tea production. Commercial tea production is mainly depended on two different varieties of *C. sinensis*, i.e., China type (*C. sinensis* var. *sinensis*) and Assam type (*C. sinensis* var. *assamica*) (Banerjee, 1992; Ariyarathna et al., 2011; Chen and Chen, 2012; Mondal, 2014).

Tea plant is an evergreen, perennial, and slow growing shrub. Tea flowers are bisexual, actinomorphic, bracteolate, hypogynous, pedicellate or sessile, and either solitary or in clusters formed at cataphyllary axils. The sepals are usually 5-6 in number, imbricate, persistent, leathery, ovate or orbicular, glabrous, and green in colour. The petals are 5-8 in number, basically connate, broad oval to sub-orbicular, and generally white in colour. The androecium formed with numerous stamens, varies between 100-300 per flower, arranged in two whorls, often connate at the base, filaments of inner whorl of stamen shorter and fewer in number, outer filament whorl longer and more numerous, 8-13 mm long, and united at the petal bases (Kapil and Sethi, 1963; Banerjee, 1992; Ariyarathna et al., 2011; Mondal, 2011; Chen and Chen, 2012; Mondal, 2014).

Flowers are the sexual reproductive organs of angiosperms. The male reproductive unit of flower, i.e., stamen is made up of the anther and filament. Pollen grain is the male gametophyte in flowers and its major role is to deliver the male gametes for the double fertilization in sexual reproduction. Development of male gametophyte involves a series of events to produce and release mature pollen grains from the anther. Anther and pollen ontogeny has been studied as one of imperative sections in angiosperm embryology because of its fundamental importance in plant reproduction (Koltunow et al., 1990; Goldberg et al., 1993; Lersten, 2004). In addition, the comprehensive knowledge of the anther and pollen ontogeny is indispensable as it has been applied in diverse scientific disciplines, i.e., plant taxonomy, evolution, phylogenetics, plant breeding programs, and biotechnology (Scott et al., 1991; Sathyapala et al., 1998; Lersten, 2004, Luna and Ochoterena, 2004; Zou et al., 2013). Further, Scott et al. (1991) perceptively reviewed the applications of the anther and pollen in molecular biology rather than in plant reproduction. However, according to the perusal of literature, the studies on anther and pollen ontogeny of *C. sinensis* are surprisingly meager and quite a few studies on palynology of *C. sinensis* have been reported (Kapil and Sethi, 1963; Kato and Shimura, 1970; Sathyapala et al., 1998; Song et al., 2008).

Hence, there is an obvious scanty of information on anther and pollen ontogeny of tea family, even though several other studies also have been dealt with palynologycal and embryological aspects in the family Theaceae and the genus *Camellia* (Zavada and Wei, 1993; Luna and Ochoterena, 2004; Donghai et al., 2005; Ariyarathna et al., 2011; Zou et al., 2013). Consequently, the application of advanced techniques in tea plant breeding and biotechnology has been impeded.

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; Ariyarathna et al., 2011; Chen and Chen, 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 resulted from 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; Ariyarathna 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 study was conducted to evaluate the anther and pollen ontogeny and cross-compatibility in China and Assam types of tea plants.

METERIALS AND METHODS

Plant materials

Present experiments used five cultivars of *C. sinensis* var. *sinensis*, i.e., 'Asanoka' (early-plucked), 'Maryoku' (mid-plucked), 'Yabukita' (mid-plucked), 'Yutakamidori' (early-plucked), 'Okuhikari' (late-plucked), and one cultivar of *C. sinensis* var. *assamica,* i.e., 'AI-37' which were grown in a plastic greenhouse at the experimental field of Jeju National University, Korea.

Histological analysis

For anther and pollen ontogeny study three cultivars of *C. sinensis* var. *sinensis*, i.e., 'Asanoka' (early-plucked), 'Maryoku' (mid-plucked), 'Okuhikari' (late-plucked), and one cultivar of *C. sinensis* var. *assamica*, i.e., 'AI-37' were used. Nine progressive developmental stages of flowers were collected based on the morphology following Ariyarathna et al. (2011) with slight modifications (Fig. 1). The petals and pistils were removed and the whole androecium of flowers were fixed in FAA (70% ethanol: formalin: acetic acid, 18: 1: 1, v/v/v) and stored at 4^{o}C until used in the dehydration process. The dissected anthers from fixed flowers were dehydrated in an ethanol series and embedded with Technovit 7100. The transverse sections of anthers were made to a thickness of 5µm using a microtome (RM2165, Leica Co., Wetzlar, Germany) and mounted on glass slides. The semi-thin sections were stained with 0.05% alkaline toluidine blue and examined under a light microscope (Leitz DMRBE, Leica Co., Wetzlar, Germany).

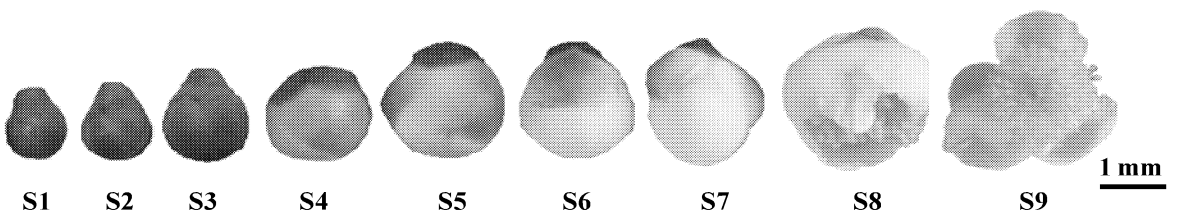

Fig. 1. Nine progressive stages of flower development of *C. sinensis*.

S1-3, round shaped flowers at the size of around 4 mm, 5 mm, and 6 mm for S1, S2, and S3 respectively with sepals and petals not differentiated, tightly furled, and dark green colored; S4-5, round shaped flowers at the size of around 8 mm and 10 mm for S4 and S5 respectively with clearly differentiated petals (light green colored) and sepals (dark green colored); S6, pre balloon stage of oval shaped and swollen flowers at the size of around 12 mm with petals white colored and still furled and sepals dark green colored; S7, late balloon stage of oval shaped and swollen flowers at the size of around 16 mm with petals white colored and still furled and sepals dark green colored; S8, petals half opened and corolla cup shaped and stamens bright yellow colored; S9, flowers soon after anthesis, with petals opened and stamens bright yellow colored and very fragrant.

Pollen quality analysis

Pollen viability and in vitro germinability was assessed at the late balloon phenophase stage (S7) of flowers (Fig. 1). Anthers were collected using fine forceps onto tracing papers laid in petri dishes and air dried at room temperature (20-25^{o}C) for 2-3 days until anthers were dehiscent and released pollen grains. Collected pollens were stored at -4^{o}C in glass tubular vial bottles with air-tight caps until used for pollen quality analysis and for artificial pollination.

Pollen viability was determined using the fluorescein diacetate-FDA test and 1% iodine potassium iodide-I_2KI test (Pok et al., 2015). Pollen was separately immersed in a trace of 1% I_2KI solution and FDA solution (200 $\mu g \cdot mL^{-1}$ FDA in 0.5 M sucrose) in eppendorf tubes following 5 min incubation at room temperature in the dark condition for proper staining of pollen grains. A drop of the 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 I_2KI and FDA tests, respectively. Six microscopic slides were used for each cultivar in each staining method. In I_2KI test, pollen grains stained with dark brown in color were counted as viable, while yellowish or unstained pollen were counted as non-viable (Fig. 2A). The viable pollen grains fluoresced brightly and non-viable pollen emitted the ghost fluorescence in FDA test (Fig. 2B). The percentage of pollen viability was determined as ratio of the number of viable grains to the total number of grains per viewed area.

In vitro pollen germination was assessed according to the method described by Yang et al. (2008). Pollens 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 hrs incubation period in the dark. Pollen grains were considered as germinated when the pollen tube length was equal or greater than the diameter of pollen grains (Fig. 3). 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.

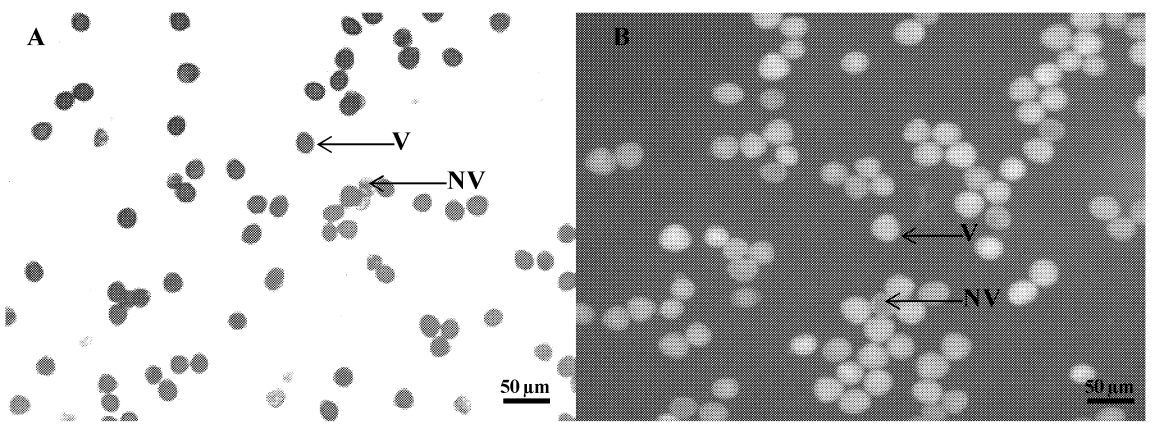

Fig. 2. Viable and non-viable pollens in I_2KI (A) and FDA (B) staining tests. V, viable; NV, non-viable.

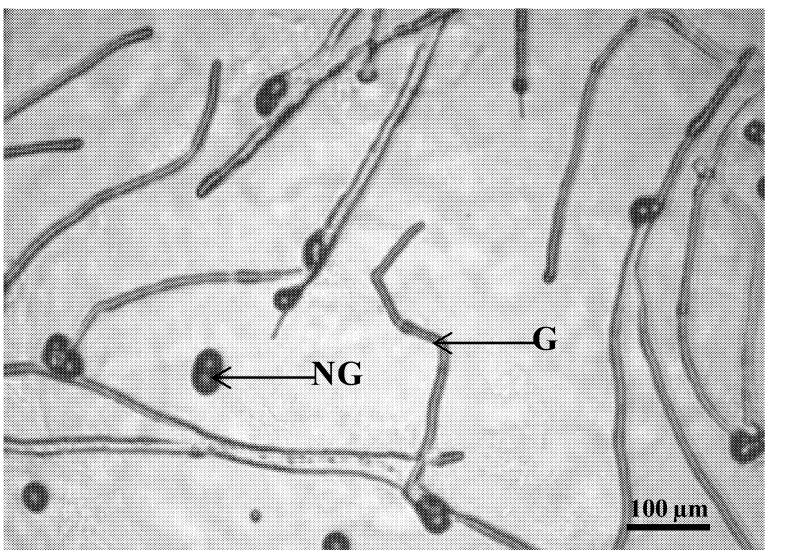

Fig. 3. Germinated and non-germinated pollen grains in germination medium. G, germinated; NG, non-germinated.

Pollination treatments

Artificial pollination was carried out in plastic green house in October, 2014 when the peak flowering occurred. *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' were used as pollen donors for artificial pollination. Late balloon phenophase (S7) flower buds (Fig. 1) 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 'AI-37' and 'Okuhikari' x 'AI-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^{o}C for 1 hr 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 K_3PO_4 for 24 hrs at room temperature in the 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 (Leitz DMRBE, Leica Co., Wetzlar, Germany).

Early fruit set

Some of pollinated flowers were left on plants 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 AND DISCUSSIONS

Anther and pollen ontogeny of China and Assam types of tea plants

In here, we used the terminology adopted by Lersten (2004) to describe the anther and pollen ontogeny of tea plant. Fig. 4 illustrated the male reproductive unit (stamen) of tea flower with two distinct portions, i.e., the anther and filament. China type and Assam type tea cultivars dominated with the dorsifixed and bilocular anthers (Fig. 4). The term dorsifixed is owing to the dorsal attachment of anthers to the apex of the filament, while the term bilocular is due to the two anther lobes and these common attributes are found in most of *Camellia* species (Zavada and Wei, 1993; Luna and Ochoterena, 2004; Mondal, 2011). Transverse anther sections of each cultivar showed two anther lobes connected by rather broad connective tissue with vascular bundles in the center (Fig. 5A). Each anther lobe consisted of two microsporangia or pollen sacs hence, dithecous. An anther consisted of four microsporangia hence, tea flower has tetrasporangiate type anther (Fig. 5A).

At the early developmental stage of flowers (S1), each pollen sac (microsporangium) of anther contained peripheral cells forming an undifferentiated wall and a mass of central archesporial cells (sporogenous cells) (Fig. 5A). Male gametophyte development of *C. sinensis* has been reported in brief by Kapil and Sethi (1963) and archesporial cells in young anthers of tea flowers also have been recognized. In the next developmental stage of flowers (S2), the archesporial cells divided and differentiated into numerous diploid microsporocytes (also called pollen mother cells, PMC or microspore mother cells, MMC) and the anther wall layers were differentiated (Fig. 5B). The archesporial cell division and anther wall differentiation are in accordance with the findings of Kapil and Sethi (1963) as these particular cells divided to form a parietal layer toward the outside and a primary sporogenous layer toward the inside. Successively, the periclinaly and anticlinaly division of the parietal layer occurred to form the anther wall. The anther wall differentiation generated a uniseriate epidermal layer, an endothelial layer, two middle layers, and a tapetum layer from surface to the interior, respectively (Fig. 5B). The ontogenetic sequence of anther wall development followed the basic type as generally found in the Family Theaceae and the genus *Camellia* (Kato and Shimura, 1970; Tsou, 1997; Zou et al., 2013). At this stage microspore mother cells were well differentiated and have dense cytoplasm and the conspicuous nuclei (Fig. 5B).

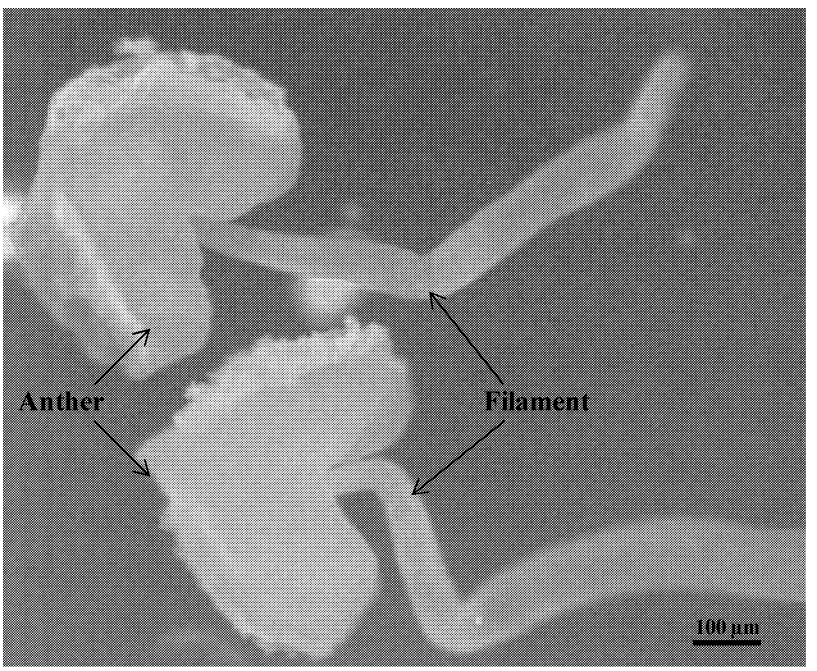

Fig. 4. Dorsifixed and bilocular anthers of *C. sinensis.* The anther is attached dorsally to the apex of the filament and possessed two anther lobes.

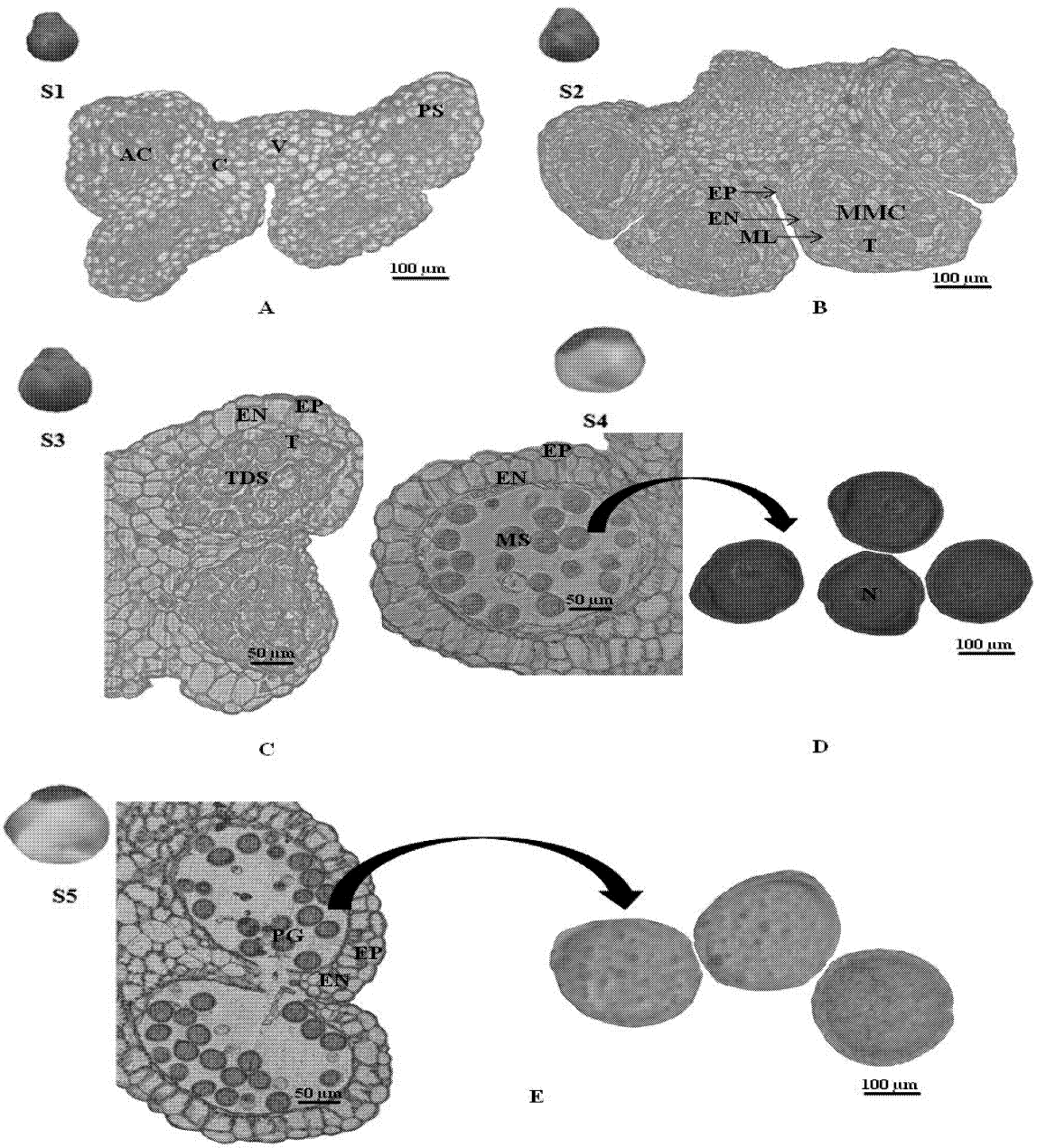

Fig. 5. Ontogenetic sequence of anther and pollen development in *C. sinensis* (L.) O. Kuntze. A, tetrasporangiate young anther with four pollen sacs (PS), archesporial cells (AC), connective tissue (C), and vascular bundles (V); B, young anther with microspore mother cells (MMC), epidermis layer (EP), endothecium (EN), two middle layers (ML), and tapetum (T); C, tetrahedral tetrads (TDS) in anther loculus; D, anthers at uninucleate (N) microspore (MS) stage with stretched epidermis (EP), fibrous thickened endothecium (EN); E, pollen grains (PG) with dense cytoplasm resulted from the mitotic division of uninucleate microspores, note that the centrally placed nucleus of microspores were disappeared and tiny granular constitutions scattered in cytoplasm of the pollen grains.

At the 3rd stage (S3) of flower development the MMC subjected to meiosis by following simultaneous cytokinesis and formed the tetrahedral tetrads which surrounded with a special callose wall (Fig. 5C). The simultaneous cytokinesis in MMC and tetrahedral tetrads formation also has been previously recorded in *C. sinensis* by Kapil and Sethi (1963) and Kato and Shimura (1970). Further, present observations were confirmed in the genus *Camellia* by Tsou (1997) in an embryological study of the family Theaceae. At the microspore tetrad stage, the anther wall was encountered with the epidermis, endothecium, and tapetum (Fig. 5C). Tapetum cells provide nutrition to the developing pollen grains and it also involves in the pollen wall formation (Scott et al., 1991; Lersten, 2004; Zou et al., 2013).

The young microspores released from tetrads were observed at the 4th stage (S4) of flower development and they were irregular in shape with a dense cytoplasm and a centrally placed nucleus (Fig. 5D). The epidermis and endothecium layers were seen at this stage. But, middle layers and tapetum layer were diminished. Since, the tapetum layer diminished at the end of the tetrads formation it has been recognized as the glandular type (also called secretory type) tapetum. Further, Kato and Shimura (1970) elucidated the similar pattern of anther wall degeneration in *C. sinensis* and *C. japonica* while Zou et al. (2013) reported comparable features in *C. grijsi*.

Subsequently, the pollen grains with a dense cytoplasm (Fig. 5E) were examined at the 5th stage of flower development (S5). At this stage the centrally placed nucleus of the young microspores was disappeared. Therefore, it is probable to presume that the uninucleate microspores were, having been subjected to mitotic division and formed two-celled pollen grains. In general, the mitotic division of uninucleate microspores formed the pollen grains with two cells, i.e., generative and vegetative cell. Regrettably, in the present study the distinctive two cells of pollen grains were not obvious. Nevertheless, the pollen grains with a large vegetative cell and a small generative cell have been examined in *C. sinensis* and some other members of the genus *Camellia* (Kapil and Sethi, 1963; Sathyapala et al., 1998; Donghai et al., 2005; Zou et al., 2013). As elucidated by Sathyapala et al. (1998) and Donghai et al. (2005), the generative cell is enclosed by the vegetative cell and located in the central part of the pollen grain. As well, we observed tiny granular constitutions in cytoplasm of the pollen grains (Fig. 5E), which have been postulated as the starch or lipid granules according to Sathyapala et al. (1998) and Lersten (2004).

As stated in those studies (Sathyapala et al., 1998; Lersten, 2004), the cytoplasm of vegetative cell in immature pollen grains contained large number of plastids with starch grains and osmiophilic globules. Hence, comparative to these facts, we can assume that the anthers at the 5th floral stage have consisted with the two-celled pollen grains. Furthermore, upon pollen germination the generative cell gives rise to two sperm cells for double fertilization, while the vegetative cell deserves as a storage organ and directs the development of the pollen tube in sexual reproduction (Sathyapala et al., 1998; Lersten, 2004). At the 5th floral stage the anther wall was composed with the stretched and flattened epidermis and fibrous endothecium (Fig. 5E). Notably, anthers at this stage showed the degeneration of the cellular septum between the two pollen sacs. Thus, young anthers of flowers in early developmental stages (S1-S4) consisted of four pollen sacs and the two pollen sacs of each side were becoming confluent owing to the degeneration of the cellular septum (Fig. 5F) at maturity stages (S5-S9).

Anthers at the pre balloon stage (S6) contained the mature pollen grains which are ready to be dehiscence (Fig. 5G). The mature pollen grain enclosed with a prominent two layered wall, i.e., inner layer termed as intine and outer layer termed as exine with three germ pores or apertures hence, tricolporate (Fig. 5H). Moreover, mature pollen grains are spherical to triangular in shape (Fig. 5H). Three germ pores and triangular shape of the mature pollen grain have been reported as general attributes in *C. sinensis* (Kapil and Sethi, 1963; Ariyarathna et al., 2011). Extensive details on the ultra structural features of *C. sinensis* pollen grain have been revealed by Sathyapala et al. (1998) using the transmission electron microscopy. The hard outer layer of pollen grains (exine) is made up of sporopollenin and it is composed of a double layer of sexine and nexine (Scott et al., 1991; Sathyapala et al., 1998; Lersten, 2004). Pollen grain exine has prominent apertures called germ pores where sporopollenin is absent. The sporopollenin is one of the most resistant organic materials which make the pollen grains extremely resistant to chemical and biological degradation (Sathyapala et al., 1998; Lersten, 2004). The inner thin continuous layer, intine is made up of cellulose and pectin (Scott et al., 1991; Sathyapala et al., 1998; Lersten, 2004).

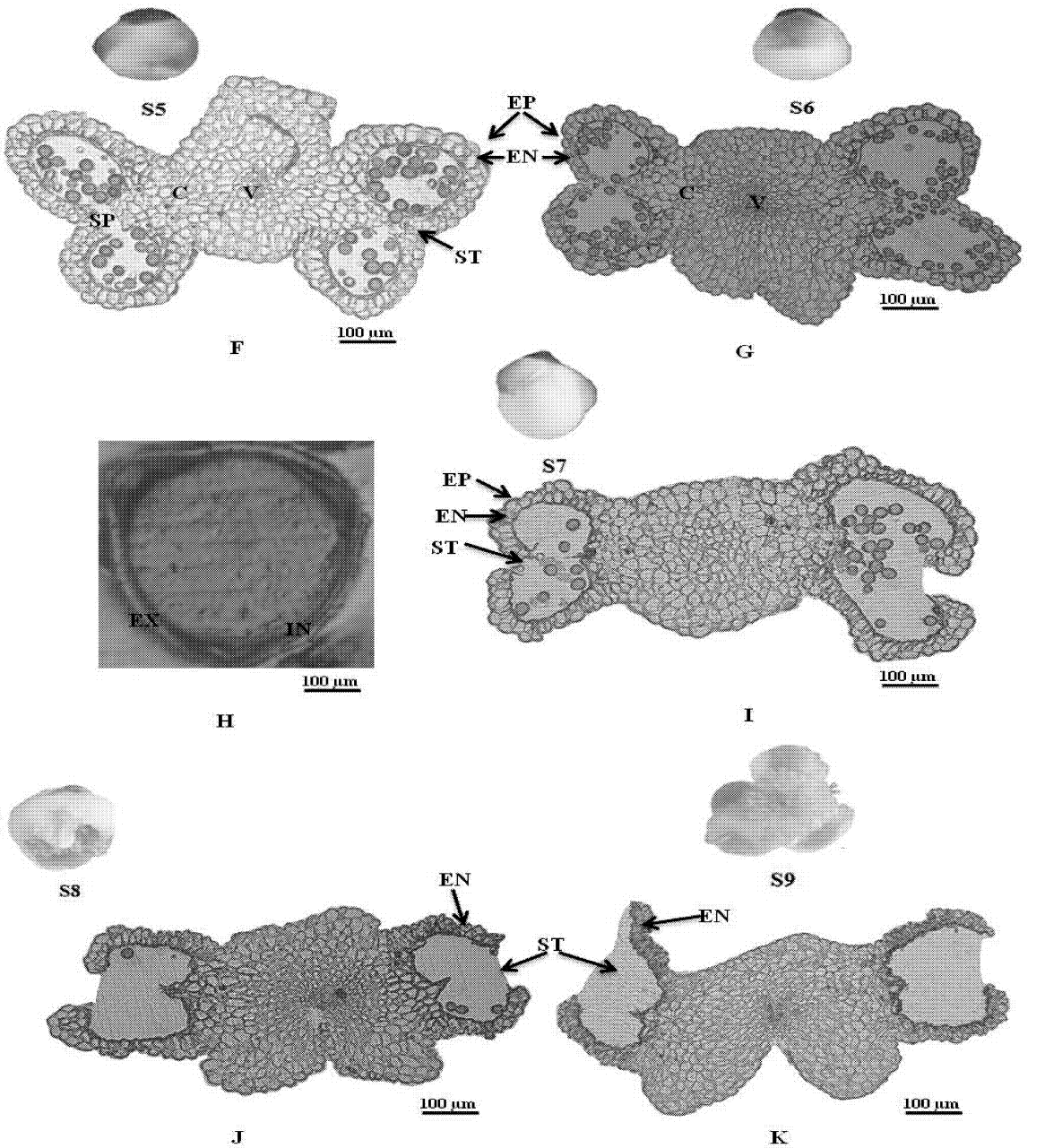

Fig. 5 (Continued). F, cellular septum (SP) degeneration in mature anther; G, anthers at the pre balloon stage contained the mature pollen grains to be dehiscence, note that the two pollen sacs of each side eventually become confluent owing to the degeneration of the cellular septum; H, tricolporate, triangular shaped mature pollen grains with exine (EX) and intine (IN); I, anther dehiscence initiated at the late balloon stage by means of longitudinal slits opened owing to stomium (ST) breakage in the anther wall; J, K, mature anther at the final stages of anthesis showing stomium (ST) separation and curved anther wall with endothecium (EN) layer.

The stretched and flattened epidermis and fibrous endothecium were observed still at the anther maturity (Fig. 5G and I). The enlargement of the anther is owing to the stretching and flattening of the epidermis and fibrous endothecium cells assist in anther dehiscence (Kapil and Sethi, 1963). At the late balloon stage (S7) anther dehiscence was taken place by means of longitudinal slits opened owing to stomium breakage in the anther wall (Fig. 5I). Anther dehiscence is the terminal step in anther and pollen ontogeny and the mature pollen grains at bicellular state were persisted to liberation. Kapil and Sethi (1963) and Zou et al. (2013) also recognized the pollen grains at two-celled stage at anther dehiscence in *C. sinensis* and *C. grijsi*, respectively. The pollen development in four pollen sacs was more synchronized. At the final stages (S8 and S9) of anthesis the anther dehiscence was fully accomplished and there was one-layered anther wall of endothecium (Fig. 5J and K).

Information generated in this study revealed an analogous anther and pollen ontogenesis in China type and Assam type tea cultivars and followed the typical stages exist in majority of flowering plants as described in the several previous studies (Scott et al., 1991; Goldberg et al., 1993; Lersten, 2004). Moreover, in spite of the cultivar type, the selected progressive developmental stages of flowers represented a good correlation with the precise stages of the anther and pollen ontogeny. The ontogenetic sequence of the anther and pollen development is exquisitely timed and choreographed and occurring in a precise chronological order that correlates with the floral bud size (Koltunow et al., 1990; Scott et al., 1991; Goldberg et al., 1993). Consequently, this correlation is exclusively supportive for the selection of floral buds with a particular developmental stage of anther and pollen for the induction of in vitro androgenesis. Hence, it is expected that the fundamental outcome of the present study is an imperative obligation for the application of the novel techniques in tea plant breeding and biotechnology, i.e., haploid plant production for obtaining the homozygous lines, pollen mediated gene transferring and cloning, and as well as in tea plant taxonomy and phylogenetics.

Cross-compatibility between China and Assam types of tea plants

In this study, *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' were used as pollen donors for 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. The data on pollen quality analysis, i.e., viability and in vitro germinability were presented in Table 1. In I_2KI test, *C. sinensis* var. *sinensis* 'Yabukita' and *C. sinensis* var. *assamica* 'AI-37' showed 88.5% and 87.5% viability percentages, in respectively. Pollen viability determined by FDA test was 82.2% for *C. sinensis* var. *sinensis* 'Yabukita' and 81.3% for *C. sinensis* var. *assamica* 'AI-37'. The in vitro pollen germination percentage for *C. sinensis* var. *assamica* 'AI-37' was 81.05% and it is for *C. sinensis* var. *sinensis* 'Yabukita' was 69.4%.

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. Heslop-Harrison et al. (1984) perceptively reviewed three general approaches for evaluating pollen quality, i.e., 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, we used I_2KI and FDA histochemical tests 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 I_2KI test has given higher values for each cultivar than by FDA (Table 1). The I_2KI 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 assessments than I_2KI test.

Table 1. Percentages of pollen viability and in vitro germination at the late balloon phenophase stage of flower buds of China type and Assam type tea cultivars.

Cultivar	Viability (%)		Germination (%)
	I_2KI	FDA	
'Asanoka'	87.9±1.6 [z]	81.0±0.4	77.0±0.9
'Maryoku'	84.2±1.4	80.8±0.8	76.7±0.8
'Okuhikari'	87.0±0.7	81.9±1.0	77.1±0.5
'Yabukita'	88.5±1.5	82.2±1.0	69.4±0.9
'AI-37'	87.5±1.2	81.3±1.1	81.0±1.3

[z] values indicate means ± S.E.

In vitro pollen germination and pollen tube growth test determines the pollen germinability on an artificial media (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 the 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, i.e., phenology of male organ and amount of pollen produced, and pollen grain traits, i.e., 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* 'AI-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* 'AI-37'can be considered as good pollinators.

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). 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, i.e., "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.

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). Four broad sites, i.e., 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; Ariyarathna 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.

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 1day after pollination in both self- and cross-pollination (Fig. 6). Wachira and Kamunya (2005) also examined the germination of tea pollen 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 hr 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 is further emphasized by Ariyarathna 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; Ariyarathna 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).

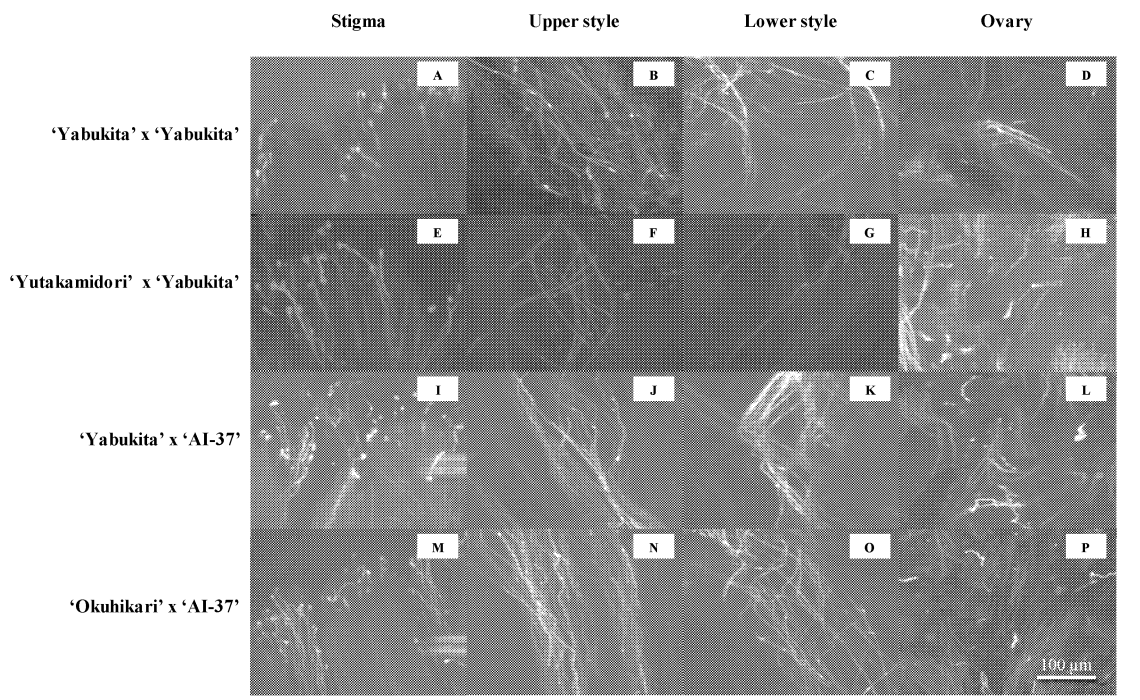

Fig. 6. In vivo pollen germination and pollen tube growth from stigma to ovary visualized using fluorescence microscopy at one day after different pollination treatments. (A–D) 'Yabukita' x 'Yabukita'; (E–H) 'Yutakamidori' x 'Yabukita'; (I–L) 'Yabukita' x 'AI-37' and (M–P) 'Okuhikari' x 'AI-37'.

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 (Fig. 6). The squashed selfed and crossed pistil samples were not clear enough to examine the pollen tubes in or near ovules. 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 hrs after self-/cross-pollination in *C. sinensis*. Supporting to our observations, Rogers (1975) also reported that by 24 hrs 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).

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. 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 previously reported studies on this subject 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 hrs after cross-pollination and 60 hrs 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 36-40 hrs after pollination, while in selfed flowers they grew uniformly and scarcely reached it by 72 hrs. Moreover, pollen tubes grew slower in styles of a different species and protruded ovules within 3-5 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 obvious morphological or structural dissimilarities in pollen grain germination and pollen tube growth were not found in self- and cross-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. Tanaka (1988) and Liao et al. (2014) reinforced our appraisal by particular studies on self-incompatibility in the genus *Camellia*. 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’) (Fig. 7). 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.

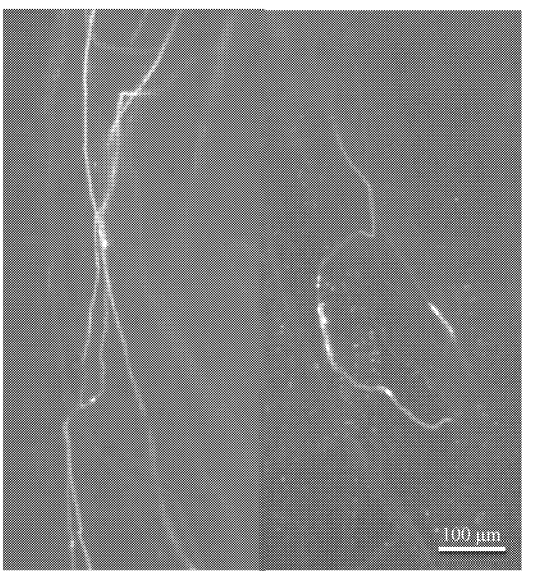

Fig. 7. Abnormal ‘zigzag’ growth pattern observed at 3 days after pollination in lower styles at very low frequency in self cross ‘Yabukita’ x ‘Yabukita’ and remote intraspecific crosses ‘Yabukita’ x ‘AI-37’ and ‘Okuhikari’ x ‘AI-37’.

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 hrs 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 percentages and fruit diameters were recorded at 3 months and 6 months after pollination (Table 2). In our observations, the cross-pollinations bore fruits whereas self-pollination failed in fruiting. 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. 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 (Ariyarathna et al., 2011). 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.

According to the depiction by Ariyarathna 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), i.e., 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 20-30% in crossed flowers and 3-10% in selfed flowers and time taken for double fertilization after pollination is 36-48 hrs and 62-72 hrs in crossed and selfed flowers, respectively. Tea fruit maturation requires 8-9 months after pollination and possesses two seeds/fruit on average with maximum of six seeds/fruit depending on parents (Ariyarathna 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 Ariyarathna et al. (2011). Percentage of pollination success was defined as the percentage of fruit set per each of pollinated flower (Ariyarathna et al., 2011).

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

Cross	Fruit set (%)		Fruit diameter (mm)	
	3 Months AP[z]	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

[z]AP-after pollination.

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* species 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 and Chen, 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 outcomes with previous reports it can be postulated that remote intraspecific hybridization might be feasible between *C. sinensis* var. *sinensis* and *C. sinensis* var. *assamica*. Supplementarily, the degree of compatibility between these hybridization species showed their genomic affinities to each other. In conclusion, the present study reveals the effectiveness of remote intraspecific cross-compatibility between China type and Assam type tea plants 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.

LIST OF CITATIONS

Ackerman, W.L. 1971. Genetic and cytological studies with *Camellia* and related genera. U.S.D.A. Tech. Bull. 1427.

Ariyarathna, H.A.C.K., M.T.K. Gunasekare, J.D. Kottawa-Arachchige, R. Paskarathevan, K.K. Ranaweera, M. Ratnayake, and J.B.D.A.P. Kumara. 2011. Morpho-physiological and phenological attributes of reproductive biology of tea (*Camellia sinensis* (L.) O. Kuntze) in Sri Lanka. Euphytica 181: 203-215.

Banerjee, B. 1992. Botanical classification of tea. p. 25-51. In: K.C. Willson, and M.N Clifford (eds). Tea cultivation to consumption. Springer.

Bezbaruah, H.P. and S.C. Gogoi. 1972. An interspecific hybrid between tea (*Camellia sinensis* L.) and *C. Japonica* L. Proc. Ind. Acad. Sci. 76: 219-220.

Chen, X., S. Hao, L. Wang, W. Fang, Y. Wang, and X. Li. 2012. Late-acting self-incompatibility in tea plant (*Camellia sinensis*). Biologia 67: 347-351.

Chen, Z.M. and L. Chen. 2012. Delicious and healthy tea: an overview. p. 1-11. In: L. Chen, Z. Apostolides, and Z.M. Chen (eds.). Global tea breeding: achievements, challenges and perspectives. Springer.

Donghai, Y., Z. Daogui, C. Zhengwu, Z. Fuyu, L. Xiangming, and L. Mengjia. 2005. Morphological studies on generative cells in pollen grains of genus *Camellia*. Chinese Agricultural Science Bulletin 21: 97-99.

Fuchinoue, Y. 1979. Analysis of self-incompatibility alleles of major varieties of tea. Jpn. Agr. Res. Q. 13: 43-48.

Goldberg, R.B., T.P. Beals, and P.M. Sanders. 1993. Anther development: basic principles and practical applications. The Plant Cell 5: 1217-1229.

Heslop-Harrison, J. and Y. Heslop-Harrison. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. Stain Technology 45: 115-120.

Heslop-Harrison, J. and Y. Heslop-Harrison. 1985. Surfaces and secretions in the pollen-stigma interaction: a brief review. J. Cell Sci. Suppl. 2: 287-300.

Heslop-Harrison, J., Y. Heslop-Harrison, and K.R. Shivanna. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. Theor. Appl. Genet. 67: 367-375.

Heslop-Harrison, Y. and K.R. Shivanna. 1977. The receptive surface of the angiosperm stigma. Ann Bot. 41:1233-1258.

Hwang, Y.J., H. Okubo, and K. Fujieda. 1992. Pollen tube growth, fertilization and embryo development of *Camellia japonica* L. x *C. chrysantha* (Hu) Tuyam. J. Japan. Soc. Hort. Sci. 60: 955-961.

Kapil, R.N. and S.B. Sethi. 1963. Development of male and female gametophytes in *Camellia sinensis* (L.) O. Kuntze. Proc. Nar. Inst. Sci. India. 29: 567-574.

Kato, M. and T. Simura. 1970. Cytogenetical studies on camellia species. I the meiosis and gametogenesis of *Camellia wabiske* compared with *C. japonica* and *C. sinensis*. Japan. J. Breeding 20: 200-210.

Kho, Y.O. and J. Baer. 1968. Observing pollen tubes by means of fluorescence. Euphytica 17: 298-302.

Koltunow, A.M., J. Truettner, K.H. Cox, M. Wallroth, and R.B. Goldberg. 1990. Different temporal and spatial gene expression patterns occur during anther development. The Plant Cell 2: 1201-1224.

Lersten, N.R. 2004. Flowering plant embryology with emphasis on economic species. Blackwell Publishing.

Liao, T., D.Y. Yuan, F. Zou, C. Gao, Y. Yang, L. Zhang, and X.F. Tan. 2014. Self-sterility in *Camellia oleifera* may be due to the prezygotic late-acting self-incompatibility. Plos one 9: 1-11.

Luna, I. and H. Ochoterena. 2004. Phylogenetic relationships of the genera of Theaceae based on morphology. Cladistics 20: 223-270.

Mondal, T.K. 2011. *Camellia*. p. 15-39. In: C. Kole (eds). Wild crop relatives: genomic and breeding resources plantation and ornamental crops. Springer.

Mondal, T.K. 2014. Breeding and biotechnology of tea and its wild species. Springer.

Mondal, T.K., A. Bhattacharya, M. Laxmikumaran, and P.S. Ahuja. 2004. Recent advances of Tea (*Camellia sinensis*) biotechnology. Plant Cell, Tissue and Organ Culrure 76: 195-254.

Muoki, R.C., F.N. Wachira, R. Pathak, and S.M. Kamunya. 2007. Potential male gametophyte competition among *Camellia sinensis* genotypes in isolated biclonal seed orchards. Afr. Crop. Sci. J. 15: 59-66.

Ozaki, Y., M. Kanda, I. Miyajima, and H. Okubo. 2003. Variation of self-incompatibility in *Camellia japonica* L. cultivars. J. Fac. Agr. Kyushu Univ. 47: 251-255.

Pok, P., E.U. Oh, K. Yi, J.H. Kang, B.Y. Ko, H.B. Kim and K.J. Song. 2015. Characterization of microspore development and pollen tube growth response to self- and cross pollination in Jeju old local citrus species. Hort. Environ. Biotechnol. 56: 225-232.

Rajkumar, S., S. Karthigeyan, R.K. Sud, R. Rajkumar, N. Muraleedaran, S.C. Das, M. Hazarika, and P.S. Ahuja. 2010. Genetic diversity of Indian tea (*Camellia sinensis* (L.) Kuntze) germplasm detected using morphological characteristics. J Cell & Plant Sci. 1: 13-22.

Rogers, S. 1975. Preliminary observations on pollen tube incompatibility in some tea clones. Tea Q. 45: 91-100.

Rosen, W.G. 1971. Pollen tube growth and fine structure. p. 177-185. In: J. Heslop-Harrison (eds). Pollen: development and physiology. London Butterworths.

Sathyapala, S.K., L.M. Guan, and T. Adachi. 1998. Ultra structural features of tea [*Camellia sinensis* (L.) O. Kuntz.] pollen. Trop. Agric. Res. 10: 254-263.

Scott, R., R. Hodge, W. Paul, and J. Draper. 1991. The molecular biology of anther differentiation. Plant Science 80: 167-191.

Simura, T. and K. Oosone. 1956. Studies on the fertilization of tea plant. Jap. J. Breeding 6: 111-114.

Song, U.S., C.J. Oh, and J.M. Park. 2008. Pollen morphology of the tea plant [*Camellia sinensis*(L.) Kuntze] in Korea. J. Kor. Tea Soc. l14: 97-110.

Takeda, Y. 1990. Cross compatibility of tea (*Camellia sinensis*) and its allied species in the genus *Camellia*. JARQ. 24:111-116.

Tanaka, T. 1988. Cytogenetic studies on the origin of *Camellia* x *vernalis* III. a method to identify the cultivars using self incompatibility. J. Japan. Soc. Hort. Sci. 56: 452-456.

Tsou, C.H. 1997. Embryology of the Theaceae-anther and ovule development of *Camellia*, *Franklinia*, and *Schima*. Amer. J. Bot. 84: 369-381.

Unal, M., F. Vardar, and O. Ayturk. 2013. Callose in plant sexual reproduction. p. 319-343. In: M. Silva-Opps (eds). Current progress in biological research. InTech.

Wachira, F.N. and S.K. Kamunya. 2005. Pseudo self-incompatibility in some tea clones (*Camellia sinensis* (L.) O. Kuntze). J. Hortic. Sci. Biotechnol. 80: 716-720.

Wachira, F.N., S. Kamunya, S. Karori, R. Chalo, and T. Maritim. 2013. The tea plants: botanical aspects. p. 3-17. In: V.R. Preedy (eds). tea in health and disease prevention. Elsevier.

Yang, H.B., S.C. Lee, S.L. Kim, M.S. Lee, J.H. Lee, T.C. Kim, Y.G. Kim, and K.J. Song. 2008. Fertilization and seed formation by artificial pollination of Tea (*Camellia sinensis*). J. Kor. Tea Soc. 14: 169-176.

Zavada, M.S. and Z.X. Wei. 1993. A contribution to the pollen morphology of *Camellia* (Theaceae). Grana 32: 233-242.

Zou, F., D.I. Yuan, J.H. Duan, X.F. Tan, and L. Zhang. 2013. A Study of microsporgenesis and male gametogenesis in *Camellia grijsii* Hamce. Adv. J. Food. Sci. Technol. 5: 1590-1595.

ABSTRACT IN KOREAN

본 연구는 차나무[*Camellia sinensis* (L.) O. Kuntze]의 중국종(*C. sinensis* var. *sinensis*)과 아샘종(*C. sinensis* var. *assamica*)에서 약 및 화분의 발생 과정 및 교배친화성을 평가하기 위해 수행되었다. 점진적인 발육단계에 따라 꽃을 채취한 다음 고정 및 염색 후 현미경하에서 약 및 화분 발생 과정을 분석하였다. 중국종과 아샘종 차나무의 교배친화성은 기내 화분 발아와 암술내 꽃가루관 신장의 형광 현미경 조사와 인공 자가수분 및 타가수분 후 착과 되는 것을 비교하여 평가하였다. 약은 네 개의 화분낭으로 이루어져 일련의 화분 발생과정(포원세포, 화분모세포, 사분자기, 일핵성 소포자 및 이핵성 성숙 화분)을 나타내었다. 성숙한 화분립은 원형에서 삼각형에 이르는 형태로 화분 외막과 내막으로 둘러 쌓여 있었다. 약벽의 발달과정은 식물에서 기본적인 일반적인 외피,하피성 내피, 중간 유조직층, 융단조직층으로 분화되었다. 약의 열개는 길이 방향의 열개선을 따라 이루어지며, 이핵성 화분이 방출되었다. 풍선상의 꽃 발달 단계에서 화분은 성숙되고 성공적인 수분 및 수정의 전제가 되는 양질의 활력 및 발아력을 나타내었다. 중국종과 아샘종 차나무에서 꽃의 발육단계에 따라서 약과 화분의 발달단계는 밀접하게 연계되어 나타냈다. 화분발아와 화분관 신장은 인공 수분 1 일, 3 일, 14 일 후 조사하였는데, 자가수분과 타가수분 사이의 화분 발아와 화분관 신장의 차이가 관찰되지 않았다. 초기착과 조사는 교배 3 개월, 6 개월 후에 수행하였는데 자가수분에서는 착과되지 않았고 타가수분에서만 착과되었다. 자가수분에서는 late-acting 자가불화합성 또는 post-zygotic barriers 가 작용하며 근연 종내 교배에서는 친화성이 높은 것으로 확인되었다. 중국종과 아샘종의 원연간 교배친화성은 교배과정에서 나타났었다. 본 연구결과는 미래의 차 육종프로그램의 개선에 중요하게 이용될 수 있을 것이다.

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my profound gratitude to Prof. Kwan Jeong Song, for affording me the chance to take out my MSc studies at Genetics and Plant Breeding Laboratory, Department of Horticulture, College of Applied Life Science, Faculty of Bio Science and Industry, Jeju National University, under his invaluable supervision. This work would not have been successfully filled out without his valuable expert advices, patient guidance, encouragements, and supports throughout this work. Further, Prof. Kwan Jeong Song is especially acknowledged for offering the financial supports to me conduct my MSc studies and facilitate my stay in Korea.

I would wish to convey my deepest gratitude to my brother-in-law, Dr. Niroshana Wickramaarachchi who completed his PhD studies at Jeju National University, my sister-in-law, Mrs. Duleeka Ranasinghe, and Dr. Isuru Wijesekara also who completed his PhD studies at Jeju National University and his wife, Mrs. Nayomi Wijesekara for giving me their enormous supports to find the opportunity for a MSc at Jeju National University in Korea.

I am greatly indebted to the members of my MSc research committee, Prof. Hoon Kang, Prof. Sang Heon Han, Prof. Sookuk Park, Prof. Young Yeol Cho, and Prof. Kwan Jeong Song for giving me supportive comments, suggestions, and fruitful discussions in all the respective areas and for evaluating and monitoring this thesis as a partial fulfillment of my degree program.

My heartiest thanks go to the Department of Horticulture, College of Applied Life Science, Faculty of Bio Science and Industry, Jeju National University, for providing me the necessary facilities with a peaceful environment for doing my academic and research works in well.

I would like to acknowledge all the present and former Korean and foreign lab mates, Ms. Eun Ui, Mr. Kyunguk Yi, Ms. Witchaya Srisook, Ms. Chang Yali, Mr. Kim Jihoon, Mr. Hong Gun-Eui, Mr. Panha Pok, Mrs. Srey Tethvoleak, Ms. Seul Gi, Ms. Ji Yeon, Mr. Seong Joon, and Mr. Jung Seok for their valuable contribution to make this attempt a success.

My heartiest gratitude goes to Ms. Eun Ui, Phd scholar for her genuine assistance given in several administrative works during my complete study period and in writing the abstract in Korean in this thesis.

A special thanks goes to Ms. Witchaya Srisook, Phd scholar; Mrs. Jirapa Austin, MSc scholar from Thailand for being with me by giving valuable advices and encouraging myself at all the time of my study period.

Special acknowledgement is extended to Prof. Park So Young, Lab of Floricultural and Biotechnology, Department of Horticultural Science, Chungbuk National University, Korea, for providing access to her laboratory for using rotary microtome for tea anther sectioning.

I also, wish to record my gratitude to my brother, Mr. H. M. S. U. Bandara, Phd scholar at New Castle University in Australia, my superlative companion, Mrs. A. N. K. Perera, Phd scholar at University of Waterloo in Canada, and my friend, Mr. Akila Wijerathna, MSc scholar at Hebrew University of Jerusalem in Israel for their immense supports and expert guidance given to me during my study period with sharing incredible assistance in finding several intellectual materials required for the completion of my thesis that I couldn't access freely and easily.

I wish to express my gratitude to all the officials at Student Dormitory Office for their kind hospitality and providing me a comfortable stay at Jeju National University.

My grateful acknowledgement also goes to my all Sri Lankan friends at Jeju National University who lent helping hands in numerous ways during my stay in Jeju.

Moreover, all other foreign students at Jeju National University are highly appreciated for their companionship accompanied with me for making my stay in Jeju as a memorable and life transforming experience.

As well, a special recognition is extended to my brother-in-law, Dr. Niroshana Wickramaarachchi and sister-in-law, Mrs. Duleeka Ranasinghe and their son, Penuja Rusara for their enormous supports and accompanies accorded during my stay in Jeju.

In addition, I wish to express sincere gratitude to my beloved teachers, since my primary education and all lecturers at Faculty of Agricultural Sciences and Management, Sabaragamuwa University of Sri Lanka who slake my craving for education.

I owe my heartfelt thanks to my fiance, Mr. W. D. W. P. Wickramaarachchi for his eternal love, continuous encouragement, and understanding during this period which greatly influenced me to complete my studies fruitfully.

Last but certainly not least, my heartfelt gratitude is devoted to my beloved parents, Mr. H. M. T. B. Herath and Mrs. U. G. Bandara Manike who have been towers of strength throughout my life, my brother, Mr. H. M. S. U. Bandara, my sister, Ms. H. M. S. N. Kumarihami, parents-in-low, Mr. W. D. Dharmasena and Mrs. S. Manike , and my dearly loved uncle, Prof. A. N. F. Perera and aunty, Prof. E. R. K. Perera for their perpetual love and affection and for been generous with their encouragement throughout the entire period of my study.

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