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Pollen production, viability and *in vitro* pollen germination of different litchi (*Litchi chinensis*) genotypes

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ABSTRACT

Litchi (*Litchi chinensis* Sonn.) is one of the important commercial fruits of the subtropical region. Its short flowering period couple with narrow genetic base serves as the major constraints in litchi genetic improvement. Pollen are known to directly influence reproductive success and genetic structure of the plant population. In this study, we compare the pollen quantity per anther, viability and *in vitro* pollen germination of two types of male flower (M1 and M2) of four different litchi genotype, viz. Shahi, China, Bedana and Kasba. Pollen quantity was evaluated by blood count method while pollen viability was assessed using acetocarmine, 2, 3, 5-triphenyl tetrazolium chloride (TTC), 2, 5-diphenyl monotetrazolium bromide (MTT) and aniline blue-lectophenol staining methods. For germination test, different concentrations of sucrose, boric acid (H_3BO_3) and agar were used. Highest pollen quantity was observed in Shahi (5292) followed by China (5022), Kasba (4775), and Bedana (4186) in the pollen from M2 flower. Acetocarmine solution (1.0 %) was most suitable dye for pollen viability test. Staining results revealed that M2 pollen were more viable than M1. Among the different media concentrations, *in vitro* pollen germination medium containing 15 % sucrose + 100 ppm boric acid + 1.0% agar showed promising results. Further assessment of the germination potential, pollen from M1 and M2 flower were incubated at different temperature and duration. Highest germination rate was observed at 25ºC with incubation period of 12 hr but maximum pollen tube growth occurs at 25ºC for 24 hr. Pollen from M2 flower had significantly higher pollen quantity, viability and germination rate compared to pollen from M1flower. Preservation and conservation of M2 pollen with high viability and germination potential for adaptation to different climatic condition of this important litchi tree fruit.

Key words: Germination rate, Litchi, Pollen, Viability

Litchi (*Litchi chinensis* Sonn.) is a sub-tropical evergreen fruit crop which needed specific climatic requirements for growth. The major litchi growing countries are China, Israel, Australia, Thailand, Taiwan, India, Vietnam, Africa Mexico and South America (Menzel and Waite 2005). World production of litchi is estimated to be around 2.11 million tonnes, with more than 95% of the area and production share of Asia. India and China account for 91% of the world litchi production but it is mainly marketed in specific areas (Singh *et al.* 2012).

Inflorescence of litchi is a compound dichasia with duodichogamy condition, producing three stages of flowers that bloom simultaneously after each other, with different degree of overlapping among them in different genotypes and climatic conditions (Menzel and Waite 2005). Flowers of the first stage are staminate (M1), followed by a second stage of pistillate flower (F) and third a stage of hermaphrodite flowers (M2). Sex ratio in litchi varies with cultivar and environmental conditions. Litchi is a highly cross pollinated crop and pollen require specific condition for stigma interaction (Menzel and Waite 2005, Singh *et al.* 2012). Therefore, information on pollen characteristics will facilitate understanding the genetics, pollen-pistil interaction, incompatibility and fertility studies, breeding and crop improvement in litchi (Gupta *et al.* 2017). So far, staining methods have been widely followed as one of the approaches in determining the quality of pollen. Pollen viability test is a rapid and easy method but they tend to overestimate the viability and germination potential of pollen grain (Gaaliche *et al.* 2013). Accuracy of different viability test varies with species or cultivars (Rodriguez-Riano and Dafni 2000). *In vitro* pollen germination is a very effective and convenient method to study the applied aspects of pollen biology (Taylor and Hepler 1997, Grover

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2016). Knowledge on the behaviour and characteristics of pollen for a specific genotypes or cultivars is important for crop improvement programs. Perusal of literature reveals a dearth of information on pollen biology. Therefore, in the present study, an attempt was made to study the pollen production, viability and *in vitro* pollen germination of litchi genotypes.

MATERIALS AND METHODS

The study was conducted on four genotypes (Bedana, China, Shahi and Kasba) maintained at ICAR-National Research Centre on Litchi, Muzaffarpur, Bihar, India. Twelve years old healthy trees were selected for the study.

Fifty anthers were collected from each stages of M1 and M2 from four genotypes before anthers dehiscence. Anthers were placed in 2 ml centrifugal tubes and then dried at 35°C under hot air oven for 24 hr until the anthers were completely dry. The tubes were vibrated to release the pollen from the anthers and vortexed for 5 min to evenly suspend the pollen in water. Pollen quantity was evaluated by the blood count method (Wang *et al.* 2015). A drop of the suspension was placed in a hemocyte counting plate to count the quantity of pollen under microscope. The number of pollen in a large square (1.0 mm × 1.0 mm × 0.1 mm in size with 400 small squares) was counted and the quantity of pollen per anther was computed as follows (Wang *et al.* 2015):

The quantity of pollen/one anther = $(2 \times n/10^{-4})/50$

where n represents the mean number of pollen in the large square, 2 represents 2 ml of suspension, 10^{-4} represents the volume of the large square and 50 represents the number of anthers (Wang *et al.* 2015).

Pollen viability was examined by different staining methods, viz. (i) 1.0 % acetocarmine solution, (ii) 2,5-diphenyl monotetrazolium bromide (MTT) (Abdelgadir *et al.* 2012, Rodriguez-Riano and Dafni 2000), (iii) Aniline blue–lactophenol staining solution (Abdelgadir *et al.* 2012, Pham *et al.* 2015). Pollen viability was viewed using Nikon eclipse 50 i microscope and was considered viable if pollens turned blue (Abdelgadir *et al.* 2012). Viable and non-viable pollen grains were counted in each field of view for a total count of number less than 100 pollen grains. Non-viable pollen grains, which remain unstained, were distinguished from viable grains. Staining percentage was determined by dividing the number of stained pollen grains by total number of pollen grains per field of view and expressed as a percentage after being normalized by angular transformation.

Anthers of M1 and M2 flowers were collected randomly from litchi genotype and before anther dehiscence. The flowers were left to dry on petri plate for 3-5 hr at room temperature. For optimization of *in vitro* pollen germination medium, the modified concentration of the composition medium of sucrose, boric acid and agar originally adopted by (Taylor and Hepler 1997) was followed (Gupta *et al.* 2017). Once the pollen germination media was optimized, pollen from each cultivar was germinated for 24 h at five temperature conditions (20°C, 25°C, 30°C, 35°C and 40°C) and 85% humidity in polystyrene petri dishes (35 × 10 mm) by scattering the pollen extracted from flowers of each cultivar onto the solidified germination medium. The temperature treatments were performed in BOD incubator. For each treatment, pollen germination was quantified in five petri dishes by counting complete fields using a Nikon eclipse 50 i Microscope until reaching at least 100 pollen grains in each plate. Total number of pollen grains and number of germinated pollen grains was recorded. Pollen grains were considered germinated when the tube length was at least twice the pollen grain diameter (Pham *et al.* 2015, Wang *et al.* 2015). Photographs were taken after staining the pollen tubes with 1.0 % acetocarmine.

All experiments were conducted in triplicate and statistical analysis was performed with JMP software version 13 (SAS 2016) using Tukey Kramer HSD test to determines the significant differences among treatment at $P \leq 0.05$.

RESULTS AND DISCUSSION

Pollen collection and pollen quantity

The quantity of pollen per anther was assessed in both M1 and M2 male flowers. It was observed that the pollen quantity increase with the developmental stage of anther and was similar among the studied cultivars (Table 1). Significant differences were also observed in pollen quantity per anther among the M1 and M2 flower in the four litchi cultivars. The highest number of mature pollen grains was recorded in M2 flower of Shahi followed by China, Kasba and Bedana before the anthers dehisce. M1 anther had low pollen quantity (40-50 %) compare to anthers collected from M2 flowers (Table 1). These results revealed that anthers of M2 flowers were the most suitable for pollen collection.

Litchi is a highly cross pollinated crop which depends on the quantity of pollen for successful pollination and higher fruit yield (Stern and Gazit 1998, Shivanna and Sawhney 1997). There was a significant difference in the quantity of pollen in M1 and M2 flowers. In addition, it was also observed that M2 anther contained higher number of pollen than M1 anthers prior to anther dehiscence. Wang *et al.* (2015) also obtained similar results in litchi cultivars Sanyuehong, Shuidong and Guiwei.

Effect of flower stage on pollen viability

The pollen viability of different M1 and M2 flowers

Table 1 Pollen quantity per anther of both stages (M1 and M2) in four litchi cultivars

Genotype	M1	M2
Shahi	2686.33±47.54 a	5292.00±68.97 a
China	2083.33±58.42 b	5022.00±69.06 ab
Bedana	1914.33±37.74 b	4186.67±82.87 c
Kasba	1536.67±67.16 c	4775.67±37.22 b

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at $p < 0.05$ by Tukey-Kramer HSD by JMP software.

under different staining methods. Results revealed that pollen viability differ significantly (≥0.05) in different genotypes with respect to stain tests and flower type (M1 and M2). Acetocarmine (1.0 %) treated anthers originating from opened flowers showed maximum viable pollen grains with different size and viability rate. Maximum viable pollens were observed in china followed by Shahi, Bedana and Kasba (Table 2). Comparatively, it was also observed that M2 flowers contained more number of viable pollen than M1 flowers in all genotypes.

Dyes (acetocarmine, aniline blue in lactophenol and MTT), which indicate the presence of cytoplasm or enzymes, respectively, were used to determine the pollen viability (Abdelgadir *et al.* 2012, Ćalić *et al.*2013, Chaudhury *et al.* 2010, Pham *et al.* 2015, Wang *et al.* 2015). M2 flowers have greater attractiveness to honeybees (Stern and Gazit 1998) that make the M2 stage more effective for pollination than M1 stage. Pollen viability was different between different genotypes after staining procedure. In this study the pollen viability of M1 and M2 flower of litchi was assessed and results similar to that reported by Stern and Gazit (1998). Acetocarmine (1.0 %) staining method is most suitable for the pollen viability test with M2 pollen being more viable than M1 pollen.

Optimization of in vitro litchi pollen germination medium

An optimized pollen germination medium has initially been developed for litchi (Chaudhury *et al.* 2010, Stern and Gazit 1998). Pollen germination on different concentration of media was observed to differ significantly. Of the different media used, results revealed that germination media containing 15% sucrose, 100 ppm boric acid (H_3BO_3) and 1.0 % agar gave the maximum number of germinated pollen grains with pollen tubes developing adequate lengths (Gupta *et al.* 2017).

Pollen germination and tube growth in vitro in different genotypes and temperature

Based on the media optimized above, pollen germination of M1 and M2 flowers of the studied genotypes, was further evaluated at five temperatures regimes (20ºC, 25ºC, 30ºC, 35ºC and 40ºC) and pollen germination percentages were determined after 24 hr in the germination medium. As presented in Table 3, all genotypes showed good pollen germination at 30ºC on the optimized medium. Significant difference was observed between M1 and M2 pollen among genotypes at different temperature. However at 10ºC, pollen germination of M1 flower did not differ significantly (data not shown). At 25ºC, there was a slight increase in pollen germination compared to 10ºC and 20ºC in both M1 and M2 pollens. Highest pollen germination was observed at 30ºC in both M1 and M2 pollen of the studied genotypes. Significantly, M1 Pollen of China gave higher pollen germination as compare to Shahi, Bedana and Kasba (Table 3). Maximum pollen germination was obtained from M2 pollens of all genotypes on optimized media. In addition, genotype China had highest pollen germination percentage in both M1 (50.33 %) and M2 (83.00 %) flowers. Above 35ºC, pollen germination reduced gradually in all the genotypes. However, germination of M2 pollen from Shahi slightly increased at 35ºC, indicating the possibility of tolerating higher temperature better than the others. It can be concluded that pollen germination from M2 flowers was higher than those of pollen from M1 flowers in all genotypes and the optimum temperature for *in vitro* pollen germination range between 25ºC to 30ºC.

Pollen germination and tube growth varies with genotype. Pollen of most species will germinate when placed in a solution of boron, calcium and an osmoticant and usually modifications in their concentrations must be performed in the different species and even among genotypes of the same species, although it provides a controlled *in vitro* condition for pollen germination (Taylor and Hepler 1997). Among them, sucrose plays a vital role both as osmoregulator and nutritive compound (Taylor and Hepler 1997). In addition, boron, calcium and other mineral salts are required in variable concentrations (Feijo *et al.* 1995, Pham *et al.* 2015). The medium developed in this work is based on a medium first developed for litchi by changing the concentrations of different salts (Chaudhury *et al.* 2010, Stern and Gazit 1998). In litchi, significantly higher percentages of germination of pollen from M2 compared to M1 flowers were reported in Israel (Stern and Gazit 1998), although differences were smaller in other regions such as South Africa (Fivaz *et al.* 1994). However, Pham *et al.* (2015) reported that pollen germination was slightly higher in M1 than in M2 flowers, while a similar trend was recorded for the two types of pollen in the different temperatures tested. Performance of pollen germination *in vitro* was low at 10 and 20ºC. This can be explained

Table 2 Pollen viability of M1 and M2 pollen of different litchi genotypes

Genotype	Aniline blue		ACC		MTT	
	M1	M2	M1	M2	M1	M2
Bedana	27.66±0.88b	79.66±0.88a	30.00±1.15c	85.00±1.52b	29.66±0.88b	77.33±0.88c
China	34.66±1.45a	82.66±0.88a	41.00±0.57b	92.00±0.57a	36.33±1.45a	87.33±1.20a
Kasba	21.66±1.20c	70.66±1.45b	25.66±0.88d	79.66±0.88c	21.33±0.88c	71.33±0.88d
Shahi	38.00±1.52a	74.33±0.88b	46.33±0.88a	88.00±0.57ab	38.33±0.88a	82.33±0.88c

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at $P < 0.05$ by Tukey- Kramer HSD by JMP software.

Table 3 Pollen germination of M1 and M2 Pollen at different temperature. Data was observed after 24 hour incubation

Genotype	20ºC		25ºC		30ºC		35ºC		40ºC	
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2
Bedana	13.66 ± 0.88b	26.66 ± 0.88b	27.66 ± 1.85bc	50.66 ± 0.88b	40.66 ± 1.76b	75.33 ± 1.45b	33.33 ± 1.20b	63.00 ± 2.08b	14.33 ± 1.20c	42.33 ± 1.45bc
China	25.66 ± 1.20a	41.66 ± 2.02a	34.33 ± 2.02ab	60.00 ± 1.15a	50.33 ± 1.20a	83.00 ± 1.15a	35.33 ± 1.85ab	74.33 ± 1.76a	21.33 ± 0.88ab	35.00 ± 1.73c
Kasba	11.33 ± 0.66b	26.33 ± 1.76b	25.66 ± 1.66c	42.00 ± 1.15c	40.66 ± 1.45b	64.66 ± 01.45c	41.00 ± 0.57a	62.66 ± 2.08b	26.00 ± 1.15a	43.66 ± 1.76b
Shahi	22.66 ± 1.45a	37.33 ± 1.20a	36.33 ± 0.88a	57.00 ± 1.15a	45.66 ± 2.02ab	77.66 ± 01.45ab	35.00 ± 1.15b	79.66 ± 1.52a	17.00 ± 1.15bc	52.00 ± 1.52a

Values are the mean of 3 (n=3). Means and SE followed by different letters are significant different at $P < 0.05$ by Tukey- Kramer HSD by JMP software.

by the origin of the crop in regions in Asia with tropical or subtropical climates (Wong 2000). Similar results were obtained in litchi (Stern and Gazit 1998, Wang *et al.* 2015) and longan (Pham *et al.* 2015, Gupta *et al.* 2017), in which the optimal temperature for *in vitro* pollen germination was also around 30ºC. However, germination of pollen at temperature above 30ºC was also observed in our study, indicating that pollen survival or possibility of pollination might vary with different sets of climatic conditions. Once the method for *in vitro* pollen germination method was developed, the protocol can be further used to study the possibilities of pollen conservation so as to bridge the flowering gap among genotypes temporally and spatially, which in turn will facilitates different breeding programs for individual genotype (Pham *et al.* 2015). Differences exist among genotypes in pollen germination at different temperatures which can be due to differences in adaptation of the genotype to temperature. Thus, this could be a useful tool to select cultivars more adapted to different climatic regions.

Conclusion

In the present study, a simple and efficient protocol for pollen germination test was developed. There was a differential effect of temperature on pollen germination in litchi, depending on the genotype.The higher viability of M2 pollens indicates greater chances of pollination in litchi cultivars which have M2 flowers overlapping with the male flowers. Determining the pollen quantity, viability and germination potential has great importance for establishing fertilization potential for selecting genotypes. Conserving pollen of the greatest viability and with greatest germination potential is of prime importance for the conservation and adaptation to different climatic condition of this valuable litchi tree fruit.

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