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EVALUATION OF POLLEN VIABILITY UNDER *IN VITRO* CONDITIONS IN TUBEROSE (*POLIANTHES TUBEROSA*)

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ABSTRACT
An experiment on pollen studies in tuberose under *in vitro* conditions was conducted in the Department of Floriculture and Landscaping, Tamil Nadu Agricultural Univeristy, Coimbatore during the year 2012- 13. The ten single genotypes *viz*.,Calcutta Single, Hyderabad Single, Kahikuchi Single, Mexican Single, Navsari Local, Phule Rajani, Prajwal, Pune Single, Shringar and Variegated Single were used for this study. Variegated Single showed its superiority in pollen viability (96.73%) and germination (98.61%) than other genotypes and it is followed by Phule Rajani (90.52% and 90.96%) under *in vitro*.High level of pollen germination was observed when PhuleRajani was crossed with Hyderabad Single (75%), Kahikuchi Single (80%), Mexican Single (94%) and Variegated Single (94%). Likewise, high level of pollen grain was noticed when Variegated Single was crossed with Calcutta Single (75%), Kahikuchi Single (57%), Mexican Single (81%), Navsari Local (50%) and Pune Single (75%) under *in vitro*. Hence, the success of hybridization by using the genotypes Variegated Single and Phule Rajani as a female parent will be highly helpful to develop new hybrids.

INTRODUCTION

The factors *viz.*, nutrition conditions of species, varieties used and environmental factors determine the germination capability of pollen (Eti and Stosser, 1988). In general, there is a linear relation between pollen viability and germination capability in many fruit species (Grigs *et al.*, 1971). *In vitro* germination of pollen has been used as powerful tool for genetical, physiological, biochemical and cytochemical studies for a wide range of plant species belonging to different families (Prajapati and Jain, 2011, Sarika Gupta and Mary Varkey Boswal, 2012).To determine the pollination potential, study was made on pollen quantity and viability, as well as on pollen germination capability and also the *in vitro* studies on pollen-pistil interaction would facilitate better understanding of the mechanism of crossability barriers in the crosses at the cellular level.

Pollen viability could be assessed by different methods like staining with nuclear or non-vital dues, by *in vitro* germination tests (Heslop - Harrison *et al.*, 1984) or by *in vitro* tests as analysing the final seed set (Razora and Zsuffa, 1986). Staining with no vital stains may be useful to determine the degree of pollen sterility but it is not useful for assessing pollen viability (Stone *et al.*, 1995). Analysing the final seed set is the most reliable method but it is not useful because it takes so much time to obtain proper information. *In vitro* germination method is regarded as a reliable test under the assumption that pollen capable of germination would be fertile pollen (Barrow, 1983). A major limitation of this test is the difficulty in achieving satisfactory germination in many species, especially in trinucleate pollen systems. It is reported that trinucleate species show some problems to develop pollen tubes in *in vitro* conditions (Mulcahy and Mulcahy, 1988).

Tuberose (*Polianthes tuberosa* Linn.) belonging to the family Amaryllidaceae is one of the important bulbous flower crops of tropical and subtropical regions. Among the commercially grown flowers in India, tuberose occupies a prime position owing to its popularity as a cut flower, loose flower, for perfumery as well as it is a potential as source of secondary metabolites. Waxy white flowering spikes of single as well as double types of tuberose impregnate the atmosphere with their sweet fragrance and because of longer keeping quality of flower spikes (Sadhu and Bose, 1973; Benschop, 1993). Tuberose flowers have long been used in perfumery as a source of essential oils and aroma compounds. Its essential oil is exported at an attractive price to France, Italy and other countries (Sadhu and Bose, 1973), as long as there is no synthetic flavour to replace it's fragrance. It is also widely grown as specimen for exhibition and cut flower.

This study was designed to determine the qualitative and quantitative characteristics of the pollen and to assess the *in vitro* pollen germination of some tuberose genotypes (Calcutta Single, Calcutta Double, Hyderabad Single, Hyderabad Double, Kahikuchi Single, Mexican Single, Navsari Local, Pearl Double, Phule Rajani, Prajwal, Pune Single, Shringar, Suvasini, Vaibhav and Variegated Single) that are presently being cultivated commercially in India.

MATERIALS AND METHODS

The present study was carried out at Botanical gardens,

Tamil Nadu Agricultural University, Coimbatore during the year 2011-2013. It is situated at 11º02" N latitude, 76º57" E longitude and 426.76 m above mean sea level. Experimental material consists of ten single genotypes of tuberose *viz.,* Calcutta Single, Hyderabad Single, Kahikuchi Single, Mexican Single, Navsari Local, Phule Rajani, Prajwal, Pune Single, Shringar and Variegated Single. The experiment was laid out in randomized block design (RBD) with three replications. The soil was brought to a fine tilth by giving four deep ploughings. Weeds, stubbles, roots etc., were removed. At the time of last ploughing, FYM was applied at the rate of 25 t ha^{-1}. After levelling, raised beds of 1.5 x 1.5 m were formed and the medium sized bulbs of 3.0-3.5 cm diameter weighing about 25 grams were planted at a spacing of 45 x 30 m which accommodates 7 plants per m^2. Uniform cultural practices were followed throughout the experimentation.

Controlled pollinations

Tuberose has the character of dichogamy. Pollen matures 2-3 days earlier than stigma receptivity (Shen *et al.,* 1987). Selected seed parents were emasculated on the day of flowering bagged immediately and pollinated 2-3 days after emasculation. After pollination, the flowers were re-bagged. Bags were removed several days later. Hybrid seeds were harvested when ripe.

Pollen viability

The pollen viability and fertility were studied by acetocarmine method as suggested by Sathiamoorthy (1973). This method allowed us to determine pollen viability and pollen fertility along flower development. The freshly dehisced pollen grains were collected from the single genotypes and placed seperately in sterilized petridishes. The pollen grains were dusted on the cavity slide followed by a drop of Acetocarmine stain on the slides. Deeply stained, normal and plumped pollen grains were considered viable while shrivelled, deformed and weakly stained pollen grains were recorded as sterile ones. Pollen fertility was assessed for three days *viz.*, first day, second day and third day of anther dehiscence and expressed in percentage.

***In vitro* pollen grain germination medium**

The following medium (Brewbaker and Kwack, 1963) was used for pollen germination.

Sucrose	15 % W/V
Boric acid	100 ppm
Calcium Nitrate	200 ppm
Magnesium Sulphate	200 ppm
Potassium Nitrate	100 ppm

Pollen viability and pollen tube growth in *in vitro* conditions

As per the method suggested by Martin, 1959, pollen viability and pollen tube growth in *in vitro* conditions were estimated. Florets were placed into FAA fixative (70% FAA- 18 ethanol: 1 formalin: 1 glacial acetic acid) at 1, 2, 4, 8, 24, 48 and 72 hours after pollination immediately following collection. After 24 h at room temperature in fixative, flowers were transferred to 70% ethanol where they were stored for up to 2 months. Prior to staining, florets were rinsed in distilled water and then softened using 8 N NaOH for 3 h. Florets were rinsed in distilled water for 30 min prior to transfer to 0.1% (w/v) aniline blue in 0.3 N K_3PO_4 for 1 h.

After staining, florets were placed on a glass slide containing a drop of glycerol and covered with 22 x 60 mm cover slip and pressed gently. The slides were observed under Olympus BX-60 microscope (Olympus America, Melville, N.Y.) equipped with fluorescent attachment using 390-420 nm barrier filter coupled with a 450 nm excitation filter. The pollen tube growth was observed and images were captured with an Olympus digital camera model E500. Pollen germination and growth of the pollen tubes through the styles were observed as bright yellow- green fluorescence. Percentage of florets in which germinated pollen was present in one or more of the stigmas was determined for each treatment. The longest pollen tube in each style was measured using ocular micrometer and an average pollen tube length estimated for each floret. Mean pollen tube length was calculated for each treatment using only those florets in which pollen germination was observed. Ovules were examined for evidence of pollen tube penetration.

RESULTS AND DISCUSSION

Pollen viability is an ability of a pollen grain to germinate and develop as a pollen tube (Gerard, 1932; Prajapati and Jain, 2011; Sarika Gupta and Mary VarkeyBoswal, 2012).Shivanna and Heslop – Harrison (1981) said that the primary determinant of pollen viability is the state of the vegetative cell membranes. It is suggested that in the partly dehydrated grain at the time of dispersal, the membranes are largely dissociated and do not form an osmotic barrier and normal properties are recovered during controlled hydration, which normally would took place on the stigma. The growth of the pollen tube can be taken as the measure of pollen viability since the non-viable pollen could not make the growth of a pollen tube. Good pod set cannot be achieved unless pollen is viable with high germination percentage. The frequency of getting fertile pollen is another factor for ensuring the fruit set. Fertile pollen along with viable pollen favour a better fruit set and consequently an acceptable yield (Sezaiercisli, 2007).In case of pollen viability (96.73%) and pollen germination (99.21%)' Variegated Single' showed its superiority over other cultivars and it is followed by 'Phule Rajani' which showed higher pollen viability (90.52 %) (Table 1). A similar study on pollen viability was earlier reported in tuberose by Seetharamu *et al.*

Table 1: Pollen viability and pollen germination in certain genotypes of tuberose

S.NO	Genotypes	Pollen viability (%)	Pollen germination (%)
1.	Calcutta Single	88.08	72.31
2.	Hyderabad Single	87.23	76.99
3.	Kahikuchi Single	79.93	63.08
4.	Mexican Single	89.11	71.27
5.	Navsari Local	88.38	78.52
6.	PhuleRajani	90.52	90.96
7.	Prajwal	39.83	22.12
8.	Pune Single	75.23	74.42
9.	Shringar	89.21	86.74
10.	Variegated Single	96.73	99.21
	SE(d)	0.16	1.65
	CD (0.5)	0.33	3.45

Table 2: Pollen tube length (microns) in certain tuberose genotypes

S.NO.	Treatments	Hours after dehiscence of anther					
		1	5	10	15	20	25
1.	Calcutta Single	6.62	10.35	18.94	23.52	29.78	36.94
2.	Hyderabad Single	35.15	70.31	151.84	216.85	389.14	446.03
3.	Kahikuchi Single	12.66	36.88	53.27	97.34	113.88	126.76
4.	Mexican Single	4.98	18.64	25.00	46.27	78.36	72.55
5.	Navsari Local	43.84	160.93	242.61	383.00	473.52	500.78
6.	PhuleRajani	76.82	98.18	210.44	452.23	625.756	726.07
7.	Prajwal	4.73	16.95	26.68	49.81	76.72	78.25
8.	Pune Single	24.34	174.67	238.07	500.18	580.41	611.55
9.	Shringar	65.00	181.50	240.53	410.63	581.55	636.32
10.	Variegated Single	118.85	520.43	839.33	1020.63	1234.95	1292.64
	SE(d)	0.08	43.84	160.93	242.61	383.00	473.52
	CD (0.5)	0.17	12.66	36.88	53.27	97.34	113.88

Table 3: Standardization of strength of NaOH and softening period for microscopic observation of pistils

Duration (hours)	NaOH (Normality)				
	6	8	10	12	14
1	IS	IS	IS	IS	ES
2	IS	IS	ES	ES	ES
3	IS	OS	ES	ES	ES
4	IS	ES	ES	ES	ES

IS – Insufficient softening; OS – Optimum softening; ES – Excess softening

(2000).

In vitro pollen germination

The composition of *in vitro* pollen germination medium will vary from crop to crop and even between the genotypes within the species. The pollen germination medium mainly contains sucrose, boric acid and other salts. The stigma used in the medium acts as an osmotic regulant and also as nutrition for pollen germination and pollen tube growth (Johri and Vasil, 1961). Voyiatizi (1995) studied the effect of sucrose, boric acid and calcium on *in vitro* pollen germination on rose cultivars. The optimum level of sucrose and boric acid enhances the germination whereas calcium has an inhibitory effect. He noticed negative correlation between calcium concentration and germination percentage.

According to Bar- Shalom and Mattsson (1977) the pollen from plants of wet stigma type is often found to germinate readily in liquid media with the appropriate osmotic balance. The technique of the pollen culture *in vitro* therefore allows the investigator to germinate the pollen grains and study the growth without interference from the tissues of the pistil. It was reported that pollen tubes *in vitro* do not grow as fast or as long as they do *in vitro* and often exhibit erratic changes in the direction (Heslop- Harrison, 1987). In the present study, the genotype differences in respect of length of the pollen tube under *in vitro* conditions were observed for 10 single type genotypes. The pollen tube length was higher in the genotype Variegated Single (Table 2). It contains higher amount of reserve food than other genotypes which may be utilized for initial autotrophic pollen tube growth.

The rate of elongation and the total length of the pollen tubes obtained *in vitro* often fell far short of the actual length requirement to reach the ovule in pistil. *In vitro* pollen tubes emerged within few minutes after 24 hours of incubation. 'Variegated Single' showed its superiority over other genotypes in pollen germination (99.21%). The pollen tube length ranged from 4.73 and 118.85 microns in one hour after incubation. The same trend was observed in 5 hours, 10 hours, 15 hours, 20 hours and 25 hours after incubations with the range of 16.95 to 520.43 microns; 26.68 to 839.33; 49.81 to 1020.63; 76.72 to 1234.95; 78.25 to 1292.64 microns respectively. The pollen tube was significantly longer in Variegated Single when compared to others(Table 2). Thus the *in vitro* studies on pollen germination and pollen tube growth although, could explain the mechanism involved in the pollen- pistil interaction at stigmatic level and in incompatible crosses. Genetic data generated earlier revealed that gynoecial factors may determine the pattern of pollen tube behaviour such as order of fertilization, rate and direction of pollen tube growth (Ottaviano *et al.,* 1980). Hence the *in vitro* studies on pollen- pistil intercation would facilitate better understanding of the mechanism of crossability barriers in the crosses at the cellular level.

Aniline blue fluorescence technique is a recently developed procedure to study the *in vitro* pollen tube growth. In this technique, pistils are stained with water soluble aniline blue and ultra violet illumination callose plug present in the pollen tube fluoresce brightly. Pollen tubes are therefore clearly distinguishable from the stylar tissues and may be readily counted and measured (Martin, 1959 and Glenda Vaughton *et al.*, 2010). Sandra (2004) described the detailed procedure for *in vitro*studies of Hydrangea pistils using aniline blue fluorescence technique. Since the number and growth of the pollen tube could be traced from such fluorescence microscopic studies and such studies were not undertaken earlier in tuberose. Hence the investigations were taken up. As a prelude to actual study, the standardization of aniline blue fluorescence technique was attempted.

Insufficient softening of pistils leaves the specimen hardy and makes difficult to observe the pollen tube growth due to differential refraction. Excessive softening leads to breakage of pistils even at a gentle pressing rendering the observation of pollen tube difficult. Therefore the optimum strength of NaOH, concentration of aniline blue, K_3PO_4 and softening period of pistil were studied and determined to be 8 N, 0.1 N and 0.3 N respectively (Table 3 and 4).

The process of pollen germination on the stigma, growth of pollen tube in the style and the penetration of the tube into one of the synergid cells with the final discharge followed by double fertilization obviously require rather detailed precise adjustment between pollen and pistil (de Nattancourt, 1977). Kallo and Chowdhary (1992) reported that such adjustment was probably an integral component of the evolution of each species. So the pollen germinaion on the stigma is the first step to indicate favourable pollen pistil interaction for successful crossing.

In the present study of tuberose crosses, pollen germination on alien stigma ranged from scale 1 to 4 which indicated the presence of wide variations in the pollen- pistil interactions within the cross compatible pollinations as well as between compatible and cross incompatible genotypes. High level of pollen germination was observed when Phule Rajani was crossed with Variegated Single in stigmatic, middle of the style and entry of ovary (310, 198 and 80) and thus points to the existence of a positive and intimate pollen- pistil interaction between these genotypes (Table 5).

The pollen grain of Calcutta Single, Kahikuchi Single, Navsari Local, Pune Single and Shringar germinated on the stigma of Variegated Single. Whereas the pollen grain of Variegated Single did not germinate on Calcutta Single and Kahikuchi Single stigma. Likewise, Kahikuchi Single, Mexican Single and Hyderabad Single pollen grains germinated on the stigma of Phule Rajani but the Phule Rajani pollen grain did not germinate on the stigma of Kahikuchi Single, Mexican Single and Hyderabad Single. It points to the presence of unidirectional interspecific incompatibility or incongruity between the two genotypes. Significant differences were also observed in pollen germination between the reciprocal crosses.

Table 4: Standardization of strength of K_3PO_4 and concentration of aniline blue for *in vitro* pollen germination studies

Aniline blue (%)	K_3PO_4 (Normality)		
	0.1	0.2	0.3
0.1	PSPF	PSPF	OSBF
0.2	PSPF	OSPF	OSPF
0.3	OSPF	OSPF	OSPF

PSPF – Poor staining, Poor Fluorescence; OSPF – Optimum staining, Poor Fluorescence; OSBF – Optimum staining, Better Fluorescence

Table 5: Number of pollen tubes at stigmatic and stylar regions and entry of ovary in single genotypes upon crossing

S.No	Cross combinations	Stigmatic region		Middle of the style		Entry of ovary	
		Range	Mean	Range	Mean	Range	Mean
1	Variegated Single x Calcutta Single	180- 250	231	116-192	129	46-65	52
2	Variegated Single x Kahikuchi Single	206- 216	213	106-159	110	40-52	45
3	Variegated Single x Mexican Single	198- 258	240	127-175	158	50-55	53
4	Variegated Single x Navsari Local	219- 278	237	127-173	140	41-61	46
5	Variegated Single x Pune Single	229- 265	254	138-170	151	37-64	47
6	Variegated Single x PhuleRajani	180- 250	231	116-192	129	46-65	52
7	PhuleRajani x Kahikuchi Single	212- 247	235	102-157	139	42-55	46
8	PhuleRajani x Mexican Single	229- 265	254	138-170	151	32-55	47
9	PhuleRajani x Hyderabad Single	202- 271	265	123-177	160	56-79	69
10	PhuleRajani x Variegated Single	296-318	310	175-212	198	73-95	80
11	Shringar x Kahikuchi Single	180- 250	231	116-192	129	46-65	52
12	Shringar x Variegated Single	198- 258	240	127-173	140	41-61	46
13	Hyderabad Single x Variegated Single	190- 290	278	135-182	179	55-82	68
14	Navsari Local x Variegated Single	197-247	231	133-172	160	37-64	16
	SE(D)	8.94		8.44		2.07	
	CD (0.5)	28.16		27.52		6.96	

Table 6: Number and percentage of ovules with pollen tube at micropylar end after 14/24 HAP in crosses

S.No	Cross combinations	Number of ovules in the ovary	Number of ovules with pollen tube at micropylar end	Percentage
1	Variegated Single x Calcutta Single	16	12	75
2	Variegated Single x Kahikuchi Single	14	8	57
3	Variegated Single x Mexican Single	16	13	81
4	Variegated Single x Navsari Local	16	8	50
5	Variegated Single x Pune Single	16	12	75
6	Variegated Single x PhuleRajani	17	15	88
7	PhuleRajani x Kahikuchi Single	15	12	80
8	PhuleRajani x Mexican Single	16	15	94
9	PhuleRajani x Hyderabad Single	16	12	75
10	PhuleRajani x Variegated Single	17	16	94
11	Shringar x Kahikuchi Single	15	8	53
12	Shringar x Variegated Single	16	12	75
13	Hyderabad Single x Variegated Single	14	8	57
14	Navsari Local x Variegated Single	14	9	64

Note: In other cross combinations among single genotypes, pollen tube did not reach ovary.

Successful fertilization involves a stepwise regulation of a series of physiological and biochemical processes and intimate pollen pistil relationship. The time required for the entry of pollen tubes into the ovule was variable depending on the distance between pollen (on the stigma) and ovule.

It was observed that for the pollen germination on the stigma, proper recognition substances are necessary. In the present study, the pollen tube length at 2 HAP in majority of the crosses was lesser than the pollen tube length measured after successful crossing at the same time interval and showed that proper recognition substances might be lacking or not strong enough to promote pollen germination immediately in unsuccessful crosses. Hence the pollen grains took longer time to germinate on the alien stigma (Gunasekaran, 1997).

In general, as the pollen start to grow, it utilizes endogenous reserves, then for further elongation the tube is dependent on stylarsecretions which break various substances down to release sugars which are ultimately used for tube wall synthesis (Gunasekaran, 1997). Arabinogalactans to be the major part of the pistil secretions that form the medium for pollen tube growth in lily.The high rate of growth of pollen tube during the initial period was evident in all successful crosses. The faster germination brought about by the strong coordination between the pollen and pistil proteins. The less number of pollen tubes was resulted in ovary regions (Table 6).

Apart from the arrest of pollen tubes at different levels in crosses, several abnormalities were noticed in the present investigation. In incompatible matings, there will be dense accumulation of callose at the tips which prevents further growth of pollen tube (Shivanna and Heslop- Harrison, 1978). The strength of callose thickening in crosses depends on the taxonomic distance between the pollen and pistil genotypes as was reported by de Nattancourt, 1977.

The most common abnormalities was the formation of knot like structures at the tip of the pollen tube. The other abnormalities include twisting and bulging at the tip, bursting of pollen tube tip and breakage of pollen tube. A similar type of malformation in pollen tube was observed in Rhododendron (Williams *et al.,* 1982) and Maize (Manickam, 1996). However, such results were not reported earlier in tuberose.

In the present investigation, there was a severe reduction in the number of pollen tubes reaching the ovary being more or less similar. The main bottleneck seems to be in the upper portion of the style and few others stop growing between mid style and ovary as in Brassica (Ockendron and Gates, 1975) and *Curcurbitapepo* (Winsor and Stephenson, 1995). Similarly Sayers and Murphy (1966) reported that in *Medicago sativa*the main determinent deciding the reduction of pollen tube number was the failure of pollen tubes to penetrate the stigma. The present study suggests that the selection pressure occuring in few crosses appears along the entire style length and particularly, a higher level of pollen tube inhibition occured at stigmatic regions in the crosses using Prajwal as one of the parent.

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