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Foraging Behavior and Role of Honeybees in *Citrus maxima* and its Impact on Fruit Production

***Abstract*: The active role of honeybees (*Apis* sp.) in *Citrus maxima* (Burm.) Merr. has been presented here with reference to pollen dispersal, pollination and frut production. The sweet scented, nectariferous flower showed late afternoon pattern of anthesis with a mean number of 18,725 pollen grains with pollen/ovule ratio 585:1. The best pollen germination (95 ± 1.4%) with a mean of 975 µm long pollen tube was observed in 20% sucrose supplemented with 100 ppm boric acid. Stigma showed maximum receptivity after 12hrs of anthesis with reference to *in vivo* pollen germination, esterase and peroxidase activity. Presence of copious esterase and peroxidase over stigma surface coincided with its receptivity. The field experimental data on the effect of netting and bagging on fruit set of *Citrus maxima* suggest that *Apis* sp. were dominant pollen vector among other flower visitors belonging to Thysanoptera, Hymenoptera and Diptera. The plant favours xenogamy showing delayed pollen and stigma receptivity. The plant attracts nocturnal flower visitors instead of its late afternoon pattern of anthesis which might be a positive selection force for the reproductive fitness and maximum fruit production. Percentage of fruit-set was considerably high in natural open flowers compared to netted and bagged flowers indicating the vital role of insects for successful pollination and suggests that fruit yields may be enhanced by introducing manageable bees together with their nesting requirement.**

Key words: *Citrus maxima*, pollination, honeybees, esterase and peroxidase activity, stigma receptivity, frut production.

In broad sense pollination biology includes pre-pollination pollen biology, pollen transfer from the anther to the stigma, pollen-pistil interaction leading to fertilization, and fruit and seed development, seed germination and seedling establishment[1]. Information on all these aspects is required not only for a comprehensive understanding of the efficiency of breeding system of a species and its evolutionary success, maintaining the structural and functional integrity of natural ecosystems, conservation, rational genetic improvement but also successful production of seeds and fruits which is necessary to meet food requirements of growing human populations and for human welfare. The abundance of choice flowers, where colour defines the intensity of attraction for bees, also influences availability of pollinators. Therefore, abundance of choice flowers within unit area around the study field is often used for assessing levels of pollination services[2,3]. As the pollinators also serve as indicators of the biodiversity pool, documentation of their diversity and distribution facilitates conservation and monitoring of biodiversity on a larger scale[4,5]. More importantly, understanding magnitude, patterns and mechanisms of pollination services is crucial for addressing the issues of food security.

Considerable research on production practices and increasing yields and quality of horticultural crops have been followed, particularly in relation to pollination biology and management practices for last few years. Usually these have been amply stated as adding nutrients, applying a number of chemicals for pests control, implementing cultural protocols demanded by the crops and the environmental conditions[6]. The studies on the effects of pollination on *Citrus maxima* production have been neglected. It is easy to understand why pollination has been neglected. It poses more difficultly as an open system than closed system research where inputs and outputs can be readily measured. The cultural practices are application of pesticides that are now reaching points of limited and in some cases it diminished returns.

It is, therefore, high time to re-examine the role of pollination as a practice on *Citrus maxima* yield. Pollination research traditionally dealt with requirements and responses of the plant, such as determining the pollination requirements, determining how those requirements are fulfilled, breeding plant lines incorporating elements contributing to increased pollination efficiency and developing cultural practices to ensure adequate pollination services. The honeybee is the primary pollinating agent of *Citrus*. Beekeepers place their colonies near *Citrus* orchards

for the delicious honey the bees store and *Citrus* specialists frequently intimate that an ample supply of bees is always in the orchards. Researchers[7-10] were taking a closer look at the pollinators, especially honey bees, and studying plant and environmental factors that affect the bee's pollinating activities, influences of bee genetics on foraging and selecting behavior, effects of the physical status of a bee colony on its pollinating effectiveness and beekeeping practices which improve pollination services in *Citrus*.

This paper deals with the current thinking on pollination biology in general and in specific about *Citrus maxima* (Burm.) Merr. production. In addition, it will examine some of the present and future possibilities about the value of the honeybees to this valuable horticultural species and this knowledge may help to provide tools that allow optimizing production and fruit with enhanced nutritional value, the ultimate goal of the *Citrus* Industry.

Materials and Methods : The study was conducted during December 2005 to November 2007 for the selected *Citrus* plants growing in the areas of Bolpur-Santiniketan Block, Birbhum district (87°41′ and 87°42′ east and 23°42′ north latitude). The flower visitors observed to pollinate the flowers were collected, preserved in our laboratory and identified using the specimens that were already identified (from ZSI, Kolkata). Flower opening were noticed following the process of Mathur and Mohan Ram[11]. Pollen grains per anther and per flower were quantified following the procedure of Mandal and Chanda[12]. Pollen morphology was described according to Erdtman[13]. *In vitro* pollen germination was studied to know the effect of different nutrients like sucrose and boric acid at different concentration separately or in combination. The fresh pollen samples were sown on grooved slides containing solution of sucrose and boric acid at different concentrations separately and/or in combination. Slides were then kept in petridishes lined with moist filter paper and observed at different time intervals to record the germination percentage and pollen tube development[14,15]. Stigma receptivity was examined by the methods[16,17], first by fixing with acetic alcohol (1:1), softening the stigmas with 4N NaOH and staining the stigmas with aniline blue. The method of Shivanna and Rangaswamy[15] was followed for esterase location over stigma surface by using alpha-naphthyl acetate as a substrate, 0.15 M phosphate buffer (pH 6.8), and fast blue B Salt. The evolution of O_2 bubbles per minute on the stigma as an indicator of peroxidase activity were recorded using hydrogen peroxide[18]. Microphotograph were taken by Zeiss (Axiostar plus) microscope at 20X magnification. Hand-pollination were performed to study the autogamy, geitonogamy and xenogamy. For geitonogamy and xenogamy, anthers were removed just before floral anthesis and bagged with butter paper immediately. The bags were removed at maximum receptive period of stigmas from these flowers and ten stigmas were pollinated with the pollen of same plant and another ten stigmas with xenogamous pollen, then bagged with butter paper to avoid other pollen contamination. For determination of apomixis, ten emasculated flowers were bagged with butter paper. Then the flowers were observed until the mature fruit development took place. After flower opening many flower visitors were found to visit the flowers for fulfilling their demand of nourishment. Mode and period of visiting, nature and behavior of flower visitors were observed visually. The flower visitors, especially different type of insects were collected with a hand-held insect catching net. Very small insects like members of Thysanoptera were collected from thrips inhabiting flowers and put into slide and mounted with a small piece of saffranin stained glycerine jelly, which consequently showed some reddish colour pollen grains adhered to the body surface of the insects and that was observed under light microscope and photograph were taken in low magnification. Pollen carrying capacity of insects were confirmed by catching and immersing the insects within distilled water taken in a petridish. Thus, the body of insects washed individually with distilled water, a drop of such water was taken onto slide and observed under light microscope, which revealed the presence of pollen grains on their body surface. Contribution of flower visitors to fruit setting were quantified by comparing fruit set between netted, bagged and open flowers. Bagging and netting of flowers were done at bud stage for ten different inflorescences per plant from same locality.

Results and Discussion : *Citrus maxima* (Burm.) Merr. is a small tree, flowers from December to February. The flowers are white, conspicuous, and sweet scented which start to open in early evening, just after flower opening anther dehiscence by longitudinal slit and nectar secretion by the fleshy disc situated beneath the ovary took place which is quite exposed and freely accessible to flower visitors. Each anther produced an average of 535 pollen grains with a mean number of 18,725 pollen grains per flower and pollen / ovule ratio 585:1 (Table–1). The pollen grains are 5-colporate, 41.5x37.5 µm, psilate with 2.3 µm thick exine; colpi 25.3 µm long, wide at mid point and tapering towards poles. Flower visitors like *Apis dorsata, A. cerana indica, A. florea, Trigona sp., Amegilla sp*. (Hymenoptera), Ants (Formicidae), Flies (Diptera), *Thrips hawaiiensis, Haplothrips ceylonicus* (Thysanoptera) were found to visit the flower for pollen and nectar (Table–2;


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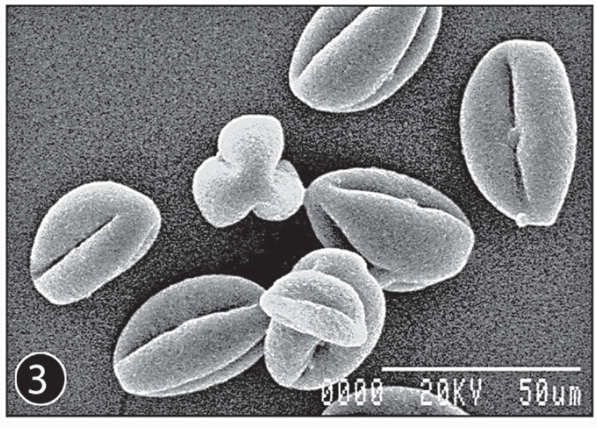
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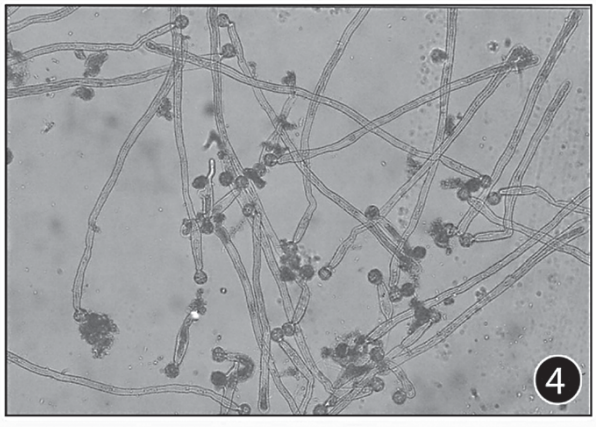
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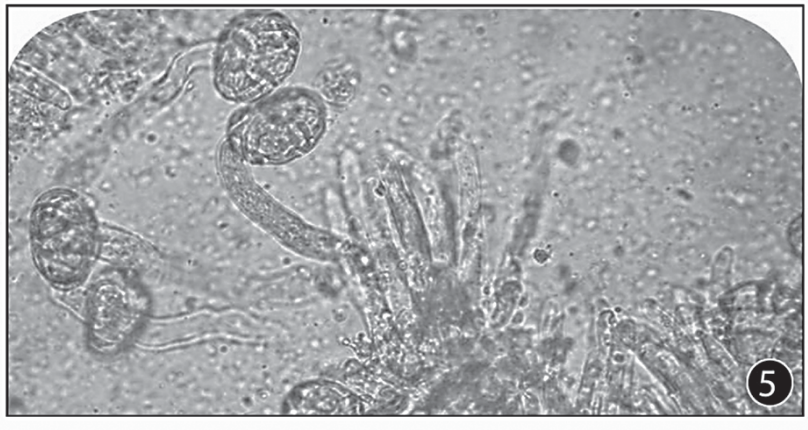
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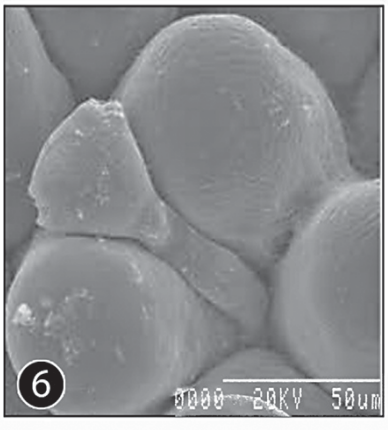
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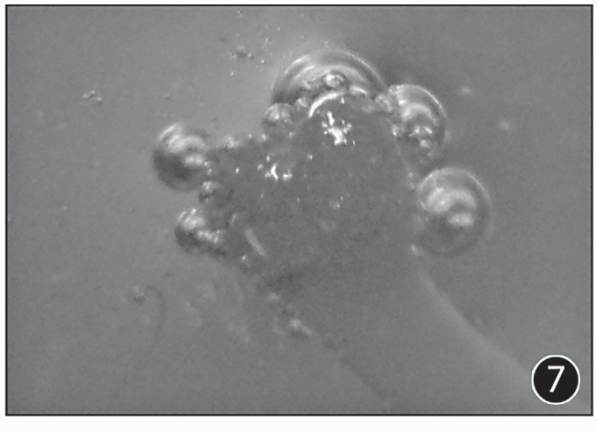
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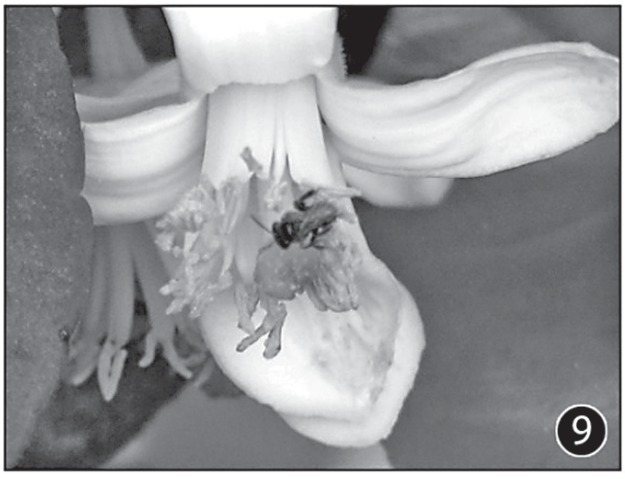

Fig.1: General habit, **Fig. 2:** Flowers, **Fig.3:** Pollen grains, **Fig. 4:** *In vitro* germinated pollen grains (LM 10X), **Fig. 5:** *In vivo* germinated pollen grains (LM 40X), **Fig. 6:** *In vivo* germinated pollen grains(SEM), Fig.7: Peroxidase activity, **Fig. 8:** Adhering pollen grains along the body parts of Thrips, **Fig. 9:** *Trigona sp.* collecting pollen, **Fig. 10:** *Apis florea* visiting the flower, **Fig. 11:** *Apis dorsata* touching stigma during forage, **Fig. 12:** *Apis dorsata* collecting nectar, **Fig.13:** Fruit-after successful pollination.

Table 1: Floral biology of *Citrus maxima* (Burm.) Merr.

Floral parameters	Characters
Flowering period	December to February
Flower type	Regular
Flower colour	White
Odour	Present
Nectar	Present
Flower opening time	Early evening
Anther dehiscence time	Just after flower opening
Mode of anther dehiscence	Longitudinal slit
Mean no. of pollen per anther	535
Mean no. of pollen per flower	18725
Pollen: ovule ratio	585 : 1
Pollen morphology	5-colporate, spheroidal, 41.5×37.5µm, 2.3µm thick exine, colpi 25.3µm long
Stigma type and location of esterases	Wet, throughout the stigma surface
Fruit-setting:	
Natural open flowers	41%
Netting flowers	13%
Bagging flowers	11%

Fig. 8-12). Members of Hymenoptera like *Apis dorsata*, *Apis cerana indica* was predominant over other flower visitors. The honeybee approaches the flowers in upright position, collects floral rewards sternotribically and carries pollen grains in huge amount. Pollination efficiency of a flower visitor is generally assessed by a consideration of the frequency of visits, pollen pick-up, foraging speed, the number of flowers foraged and the intrafloral behavior. Seed/fruit production in plants depends upon several factors and inputs; the managed pollination is one of these factors/inputs. For managed pollination of a plant, there is essential need to know about its pollinators. A plant breeder should know the knowledge about the floral biology and pollinating system without which breeding programmes are quite imposible. After considering the arrangement of anthers and stigma within the flowers, time of anthesis, visitors' frequency, their activity over the flower and the amount of adhered pollen grains of their body parts together with the possibility of such pollens deposited over the stigma (stigmatic load), it was found that honey bees is the principal pollinator of the plant.

Table 2: Enumeration of flower visitors of *Citrus maxima* (Burm.) Merr.

Name of the visitors with order/family	Visiting time	Foraging nature	Pollen adhering region
Hymenoptera			
Apis dorsata	Day	Pollen, Nectar	Dorsal region,
A. florea	Day	Pollen, Nectar	Thorasic region, Legs
A. cerana indica	Day	Pollen, Nectar	"
Trigona sp.	Day	Pollen, Nectar	"
Amegilla sp.	Day	Pollen, Nectar	"
Ants(Formicidae)	Day and Night	Nectar	
Diptera	Day and Night	Nectar	Throughout the body
Thysanoptera	Day and Night	Pollen, Nectar	
Thrips hawaiiensis	"		
Haplothrips ceylonicus	"		

Successful seed and fruit formation is largely dependent on pollination success rate which subsequently depends upon the availability of good pollinators, pollen load and its viability, stigma form and its receptivity. Studies on *in vitro* pollen germination at different time intervals after anthesis showed 70 ± 0.70% germinated pollen with a mean of 715 µm long pollen tube in 20% sucrose (Table–3). The best pollen germination (95 ± 1.4%) with a mean of 975 µm long pollen tube was observed in 20% sucrose supplemented with 100 ppm boric acid (Table–5, Fig.-4). The externally supplied sucrose maintains the osmotic pressure and act as a substrate for the pollen metabolism[19, 20]. The role of boron has been confirmed in germinating pollen and growing pollen tubes, especially in vascular plants[21,22]. Boron directly involved in pectin synthesis and attributed to enhancement in tube growth rate[22]. Though the effect of either sucrose or boric acid individually showed good results however, sucrose in combination with boric acid promoted pollen germination as well as tube development, because boron makes a complex with sugar and this sugar-borate complex is known to be capable of better translocation than non-borate, non-ionized sugar molecules[22-25]. Stigma receptivity is maximum (77 % germinated pollen grains with 569 µm long pollen tube) after 12 hours of flower opening (Table-6; Figs.-5 & 6). Esterase secretion was found to be predominant at the time of higher receptive period. The presence of copious esterases and peroxidase over broad stigmatic surface coincided with its receptivity. Prominent presence of esterases and peroxidase was observed during higher receptive period of stigmas**.** Based on *in vivo* pollen germination, non-specific esterase expression and peroxidase

activity indicates that stigmas of *Citrus maxima* have longer receptive period. The percentage of fruit set were 41%, 13% and 11% in natural open, netted and bagged flowers respectively. The observation on hand pollination revealed that the fruit set were 25%, 45% and 80% in autogamy, geitonogamy and xenogamy respectively.

Sweet scented nectariferous low pollen producing flowers of *Citrus maxima* attracted the flower visitors of Thysanoptera, Hymenoptera and Diptera. The flowers showed greater number of pollen grains per ovule, which reflects the positive direction towards xenogamous selection[26]. The floral structure, orientation of petals and position of anthers as well as stigma support the out-crossing tendency and may overcome the barrier of selfing. This finding may lead towards the generation of genetic variation and vigorous size of *Citrus maxima* fruit. Under natural condition, McGregor[6] has shown, that the cross pollination of *Citrus* yielded large size fruit and higher productivity than that of inter-selfing or geitonogamy under field condition. Among the different insect flower visitors, the visual observation suggest that the *Apis* spp. (*Apis dorsata*, *A. florea*, *A. cerana indica*) were predominant which may be due to the bee-floral feature of *Citrus maxima* and abundant amount of floral nectar. As the pollen output is less, so it is presumed that nectar is chief attractant for flower visitors, especially honey bees, although flower odour might not be neglected. Critical analysis on the determination of actual pollinator component and isolation of primary forage matter (nectar or

Table 3: Effect of sucrose on *in vitro* pollen germination of *Citrus maxima* (Burm.) Merr.

Concentration	After 1 hr.		After 3 hrs.		After 6 hrs.	
	Germination (%± S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length (µm)
5%	15±1.16	195	20±1.27	247	20±1.27	312
10%	28±0.41	221	35±0.86	260	35±0.86	377
15%	44±1.69	299	45±0.78	403	45±0.78	650
20%	62±0.95	325	70±0.70	455	70±0.70	715
25%	50±0.62	260	55±0.66	429	55±0.66	676
30%	38±0.99	208	45±1.23	312	45±1.23	585
35%	20±0.78	195	22±0.90	299	22±0.90	312

Table 4: Effect of boric acid on *in vitro* pollen germination of *Citrus maxima*

Concentration	After 1 hr.		After 3 hrs.		After 6 hrs.	
	Germination (%± S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length (µm)
50 ppm.	25±1.61	105	30±0.72	195	30±0.72	208
100ppm	33±1.32	130	35±0.77	260	35±0.77	455
200ppm	42±1.72	312	45±1.60	325	45±1.60	533
300ppm	22±0.79	260	24±0.99	299	24±0.99	364
400ppm	10±0.54	195	11±0.81	208	11±0.81	221

Table 5: Effect of sucrose and boric acid on *in vitro* pollen germination of *Citrus maxima*

Concentration	After 1 hr.		After 3 hrs.		After 6 hrs.	
	Germination (%± S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length(µm)	Germination (%±S.E)	Mean tube length(µm)
20% + 50ppm	62±1.73	325	63±1.74	520	63±1.74	676
20% + 100ppm	92±1.60	364	95±1.40	689	95±1.40	975
20% + 200ppm	76±1.74	312	77±1.19	520	77±1.19	832
20% + 300ppm	55±0.96	299	55±0.94	390	55±0.94	637
20% + 400ppm	39±0.61	273	40±0.64	455	40±0.64	520

Table 6: Stigma receptivity of *Citrus maxima*

Time after flower opening	After 1 hr.	After 2 hrs.	After 6 hrs.	After 12 hrs.	After 24 hrs.	After 48 hrs.	Drooping stage
No. of stigma observed	10	10	10	**10**	10	10	10
Averg. no. of pollen over stigma	226	112	450	**400**	390	425	310
Averg. no. of germinating pollen over stigma	106	175	334	**310**	245	165	144
% of germinating pollen grains	47	64	74	**77**	63	39	35
Pollen Tube length (mm)	298	326	495	**569**	443	325	175
Esterase expression	+++	+++	+++	**+++**	+++	++	+
Peroxidase activity	+++	+++	+++	**+++**	+++	++	+

+++ : High, ++ : Moderate, + : Low, – : Absence

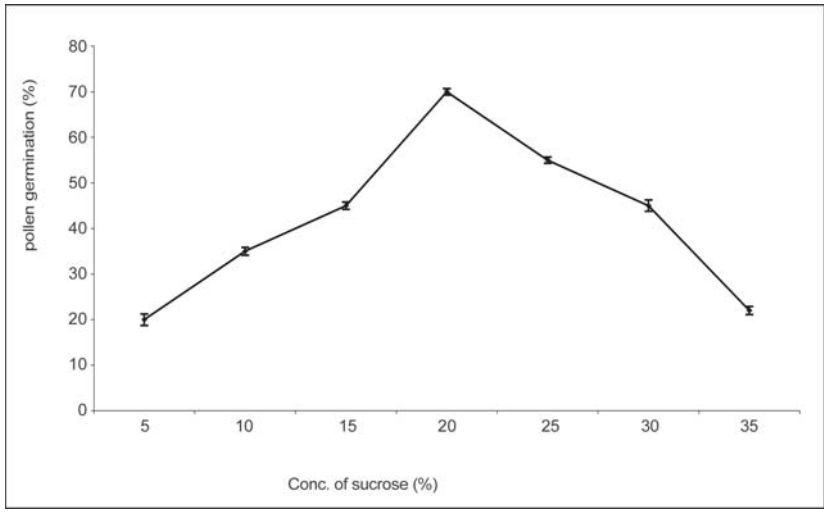

Fig.14a: Effect of sucrose on *in vitro* pollen germination

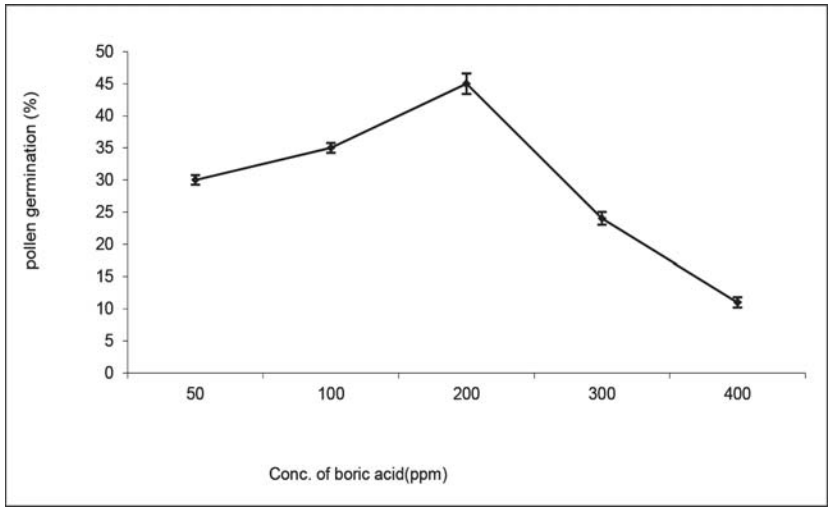

Fig.14b: Effect of boric acid on *in vitro* pollen germination

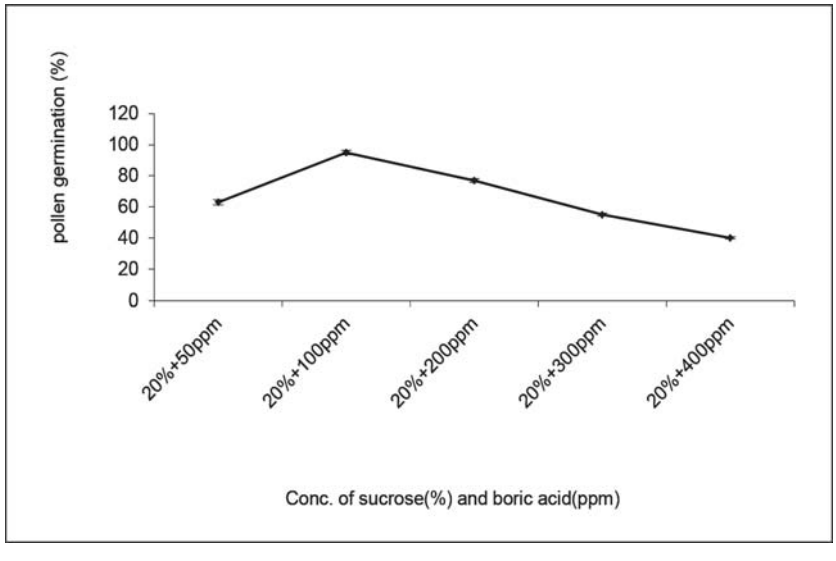

Fig. 14c: Effect of sucrose and boric acid on *in vitro* pollen germination

pollen) is yet to be studied but we have been able to determine the role of flower visitors on fruit set data (Table-1). Minor role of thrips (Fig.-8) in pollinating *Citrus maxima* is indicated through less percentage of fruit set in netting condition, where as natural open flower, which were exposed to all pollinators indicating the role of large body flower visitors (*Apis* spp., Ants and Diptera) which showed comparatively greater percentage of fruit set. Krezdorn[8] reported the major role of large body insects particularly *Apis* spp. for greater number of fruit set in different *Citrus* species. Role of flower pollinating honey bees has a paramount significance for the increase in fruit number, size and quality of *Citrus* spp[7,10]. The strong xenogamous nature is followed because of higher pollen ovule ratio. Xenogamy is predominant over here, probably due to coherent pollen competition for sperm discharge and less frequency of pollen abortion. Cruden[26] reported that xenogamy is favoured by high pollen ovule ratio and pollen competition. Besides, the plant showed late afternoon and evening pattern of anthesis but pollen and stigma receptivity continued upto next day. This is also an adaptive feature in favour of cross pollination in *Citrus maxima*. The best pollen germination (*in vitro* and *in vivo*), greater stigma receptivity and high esterase expression were noticed in first day after anthesis which could be attributed to inhibit selfing, or favour cross nature, or to attract diurnal visitors only. This is also a positive selection pressure imposed upon the plants to overcome the problem for hawkmoth (nocturnal visitors) for effective pollination of *Citrus maxima*. Moreover, this work envisages the role of diurnal insects (except ants and thrips) on plants fitness in anticipation with pollination and fruit set. The plant showed delayed pollen and stigma receptivity to promote cross pollination, largely to further focus on the predicted xenogamous theory versus increment of fruit set in *Citrus maxima*.

Fig.14d: Stigma receptive period of *Citrus maxima*

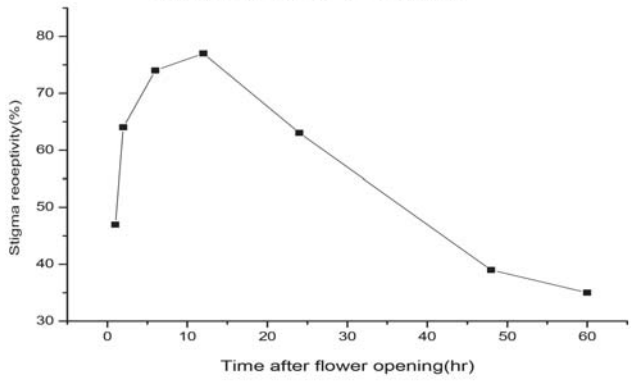

Fig.14d: Stigma receptive period of *Citrus maxima*

Conclusion : The declining horticultural productivity can be attributed to a number of factors, but pollination plays a crucial role. One can go for use of improved agricultural technologies, such as the use of quality seed, high yielding varieties, good agronomic practices, but without pollination, neither fruit nor seed will be formed. Pollinator scarcity is the main factor responsible for inadequate pollination which can be overcomed by conserving manageable species of honey bees' populations. The study indicating the vital role of insects for successful pollination suggests that fruit and seed yields may be enhanced by introducing manageable bees together with their nesting requirement. Promoting use of beekeeping for pollination of horticultural crops will be of benefit to both the beekeeper and farmer. As the bees are manageable, the possibilities to exploit its potential for crop pollination, honey making and better agricultural economy must be explored. It is expected that the present findings will speed up the invention and application of novel cultural practices and help in the development of more adapted varieties.

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