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## Research Article

### Floral, reproductive biology and morphological variation in annatto (*Bixa orellana* L.)

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#### Abstract

The present study aimed at evaluating the genetic variability of different germplasm and comparative effectiveness of morphological descriptors, floral and reproductive biology of three CPTs of annatto viz., Tamil Nadu (green fruited), Kerala (red fruited), Karnataka (dark red fruited) at department of Forest Biology and Tree improvement, Forest College and Research Institute, Mettupalayam during 2017-19. A record on sequence of flowering ascends along the latitudes. The sequence of flowering is top to bottom (basipetal) that is the development, maturation or opening from the apex towards the base in the sequence. It commenced from the southern side to the south eastern side. The colour of the flower is whitish pink in Tamil Nadu, moderate pink in Karnataka and dark pink in Kerala and this floral colour polymorphism suggests that pollinators (mainly bumble bees and ants) are the primary selective agents which influence flower colour. The stain I-KI showed 95 % pollen viability and it is found that the viability of the pollen grains decreased after four hours from pollen dehiscence and tricolporate pollen was observed in annatto. The observation on fruit set indicated that bixa plant can have both self- and cross-pollination. Fruit maturity takes place 80 – 95 days counting from the day of pollination to fruit dehiscence. The shape of the fruit resembles rounded oblong pyriform. Maximum seed production was recorded in the red fruited (collected from Karnataka) (113.12 g) plant followed by the dark red fruited plant (Kerala) and the green fruited variant (TN).

#### Key words

Annatto, reproductive biology, pollen viability, anthesis time, pollination

#### INTRODUCTION

Natural dyes are biodegradable, non-toxic, aesthetically appealing and may serve a better alternative to generate employment and utilize the wastelands. Natural dyes are obtained from plant, animal and mineral resources. The main source of obtaining raw material for natural dye is through cultivation and collection. The pigment content in natural dye source is very low and due to that huge raw material is required. For the purpose of dye extraction, very few natural dyes such as indigo, marigold, henna, etc., are cultivated in India. Lots of species with good dye content are available in forests which are to be brought under cultivation. It is estimated that around 500 species are potential natural dye sources do exist in India but of which only less than 10% is used as natural dye source. *Bixa orellana* L. is a perennial tree native to the Neotropics (Rivera-Madrid *et al.*, 2006), also known as annatto or achiote in Mexico and urucum in Brazil. Bixa is the only

genus of Bixaceae and includes six species (Carvalho *et al.*, 2005), with *B. orellana* being the most important, used in the food industry as a natural red dye constituting bixin and nor bixin carotenoids found in the seed coat.

Rivera-Madrid *et al.* (2006) studied the *in vitro* germination using bixa pollen, an average pollen viability is about 86%, and pollen longevity declines by about 50% 24 h after release from the anther. Significant differences in carotenoids and bixin contents in mature seeds were observed between the studied annatto variants such as white, purple and pink petals. Besides food-industrial use, studies have pointed out some medicinal properties of its natural pigment such as antigenotoxic and pro-vitamin A like activity (Karchuli and Ganesh, 2009). Roychowdhury *et al.* (2011) studied the floral characteristics in *Dianthus caryophyllus* in temperate climates, is worldwide popular

as cut-flowers for its variegated petal's colour and reported that the development of this cultivar with more desirable floral characteristics and higher productivity are also very much important. Both morphological and/or genetical characteristics were considered using different laboratory methods and reported that peculiar morphological architectures of leaf stomata in *Dianthus* at different concentrations of three potent chemical mutagens were analysed on the basis of their Scanning Electron Microscopy (SEM) images which is more informative than the classical approach. Joseph *et al.* (2012) presented studies for some reproductive characters of an Indian population of *B. orellana*, showing its pollen morphology and viability. There is no data for self-compatibility or apomictic fruit for annatto or for other *Bixa* species. Rivera-Madrid *et al.* (2006) presented a methodology for an artificial pollination in *B. orellana* and

data floral biology. Very little research, however, has been carried out on the sexual reproduction of *B. orellana*, and consequently, many aspects of its reproductive biology are only partially unveiled. There is an urgent need to develop high yielding varieties, precision cultivation techniques and value addition techniques in the natural dye yielding plant species *viz.*, Annatto (*Bixa orellana* L.) and the farmers should be encouraged to cultivate more profitable natural dye yielding plants instead of water intensive commercial crops. As the annatto dye and seed yield vary from sample to sample and plant to plant (Valdez-Ojeda *et al.*, 2008), and due to its economic potential, to accelerate the breeding programs, the present study aims to characterize the floral, reproductive biology and morphological variation of annatto followed by breeding of specifically selected parent plants.



**Fig. 1.** Field view of different germplasm assemblage of annatto

## MATERIALS AND METHODS

The field studies were carried out in the *bixa* field (Fig.1), Department of Forest Biology and Tree improvement, Forest College and Research Institute, Mettupalayam, Tamil Nadu which is situated at 11°09'N latitude and 76°56'E longitude. The location lies 300 m above MSL. The study was conducted in a germplasm assemblage and focused on comparative effectiveness of morphological descriptors and the floral and reproductive biology of three CPTs of annatto collected from Tamil Nadu (green fruited), Kerala (red fruited), Karnataka (Dark red fruited) (Fig.2).

Five trees from each CPTs were tagged for observation. During flowering and maturation the data on floral and morphological traits *viz.*, sequence of flowering, number of flowers per inflorescence, length of inflorescence, inflorescence type, colour of the flower, shape of petal, the number of rachis per inflorescence, time of anthesis, time of maximum insect visit, style length, length of the stigma,

pollen viability test, the number of primary branches, the number of secondary branches, the number of flowers per inflorescence, the number of fruits per inflorescence, type of fruit, fruit length, fruit colour, fruit width, length of the leaf, width of the leaf, shrub height, collar diameter and single tree yield were recorded.

Unopened flowers were collected at balloon stage from all sides of one tree. Petals and sepals were separated and anthers were isolated from flower buds and placed on a black paper under an incandescent lamp on a table overnight. The next day, the pollen grains were collected in a small glass vial. Pollen viability was estimated using four staining techniques, IKI (iodine potassium iodide) (Baker and Baker, 1979), TTC (2, 3, 5-triphenyl tetrazolium chloride) (Norton, 1966), 1% TTC (0.2 g TTC and 12 g sucrose dissolved in 20ml distilled water) and Saffranin. 1 % TTC was used in the first step and the mixture was dropped on a microscope slide and the pollen spread

with a slim brush and covered with a cover slip. Pollen viability counts were made after two hours; then pollen was placed in TTC solution. Pollen grains that stained orange or bright red colour were counted as viable. In the third staining method, 1g potassium iodide and 0.5 g iodine were dissolved in 100mL distilled water for the IKI solution. Pollen viability counts were made five minutes after pollen was placed on an IKI solution. Pollen grains stained dark (dark red or brown colour) was counted as alive. In the fourth staining method, 1 g safranin and 40 ml 95% ethanol dissolved in 60 ml distilled water for the safranin solution.

Stigma receptivity was evaluated using stigmatic peroxidase activity according to the method of Kearns and Inouye (1993). Intact styles of emasculated flowers were placed on a glass slide in a drop of 3% hydrogen peroxide and covered with a cover slip. Stigmas that produced bubbles within 2–3 min were considered receptive.

Selected flower buds were emasculated on the day before blooming. The anthers were carefully removed without damaging the receptor flowers. Synchronously opened flowers in a single panicle were removed retaining one in each panicle. The flowers were then covered with butter



**Fig. 2. Morphological features of the plants used in the controlled breeding experiments**

a. Green-fruited plant (TN) with a bunch of capsules. b. Single flower of green-fruited plant. c. Fruit of green-fruited plant. d. Red-fruited plant (Karnataka) with a bunch of capsules e. Single flower of red-fruited plant. f. Fruit of red-fruited plant. g. Dark red fruited plant (Kerala) with a bunch of capsules. h. Single flower of dark red fruited plant. i. Capsule of dark red fruited plant

paper bags and tied up. The effective time for pollination was determined based on the stigma receptivity and pollen viability records. The pollen grains were collected using a fine brush and were introduced into the receptive stigma by gently touching with the brush. The receptor flowers were then covered by closing the bag and tagged. Both self and cross-pollination experiments were carried out and the data were collected to find out the mode of pollination.

## RESULTS AND DISCUSSION

In annatto, the inflorescence is a terminal panicle (compound raceme) with several flowers (Fig.3a). Seven days after the initiation of inflorescence the central buds were labelled and considered for later studies. The sequence of flowering is top to bottom (basipetal) that is the development, maturation or opening from the apex towards the base in the sequence. Flowering commenced from the southern side to the south eastern side. Barret *et al.* (1999) have reported such sequence of flowering on plant characteristics and phenology in *Azadirachta indica*, *Pongamia pinnata* and *Ailanthu sexcelsa*. Morphological diversity and variations pigment yield were reported Akshatha *et al.* (2011).

The time of flowering is determined by endogenous genetic components as well as various environmental factors, such as day length, temperature, and stress. Anthesis occurred during the morning hours. Based on the data obtained from 15 mature buds per tree, the mean anthesis time starts from 6.00 pm and completed around 7.30 am the next day (Fig. 3d). Sareen and Banwara (2001) found that in *Cassia siamea*, flowers open from 5.00 am to noon with peak anthesis from 9.00 to 10.00 am. In annatto, the time from the manifestation of a flower bud to an opened flower ranged between 28 and 30 days and the annatto flower opens at dawn (4.00 am) and anther dehiscence has occurred by 9.00 am (Nisha Joseph *et al.*, 2012). Ambient temperature influences flowering time by affecting the rate of growth and development throughout the plant life cycle (Jagadish *et al.*, 2016)

*Bixa orellana* has several cultigens characterized by distinct phenotypic features such as shape and color of capsules and flowers. In the recent past, morphological variation based on leaf, flower and fruit characters of *B. orellana* was reported (Akshatha *et al.*, 2011). The conventional knowledge of floral color polymorphism suggests that pollinators are the primary selective agents which influence flower color. The qualitative floral characters considered for the study were petal colour, anther colour, filament colour, style colour, stigma colour and fruit colour (Fig 2 and 3). The colour of the different floral parts was determined using the standard colour chart (Wilson, 1941). In this study, the variation in the flower colour and fruit colour were observed from the three CPTs. The colour of the flower is white in Tamil Nadu source, moderate pink in Karnataka source and it is dark pink in Kerala source (Fig.2). In addition to floral colour, pollinator may also show preference for corolla shape

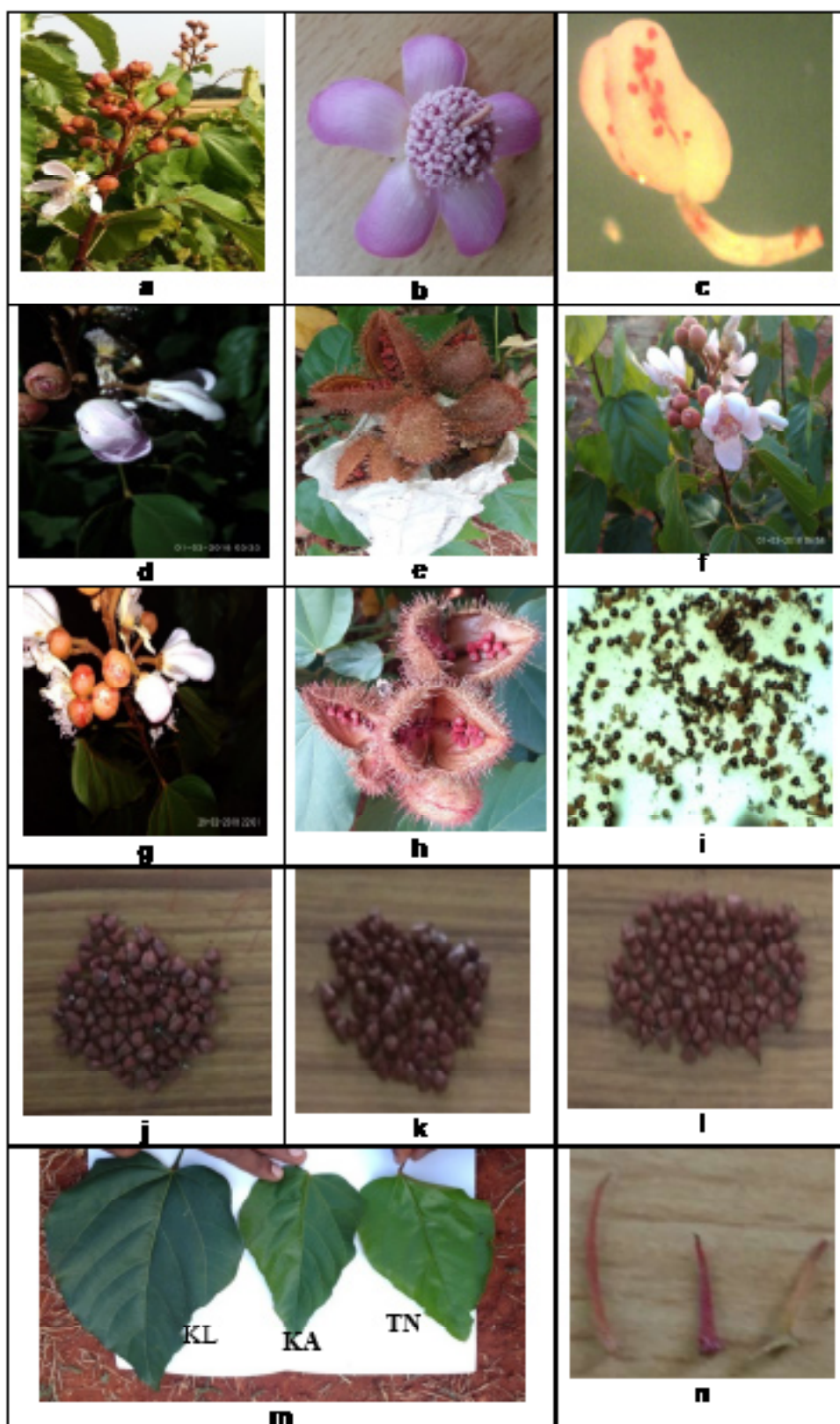
(Gomez and Perfectti, 2010), floral odour (Raguso, 2008) and flower size (Conner and Rush, 1996) to contribute to the reproductive success of flowering plants.

During reproductive surveys several pollinators were observed in the *B. orellana* flowers, mainly bumble bees (*Bombus* sp.) and ants. The *Bombus* bees presented a high visiting activity since early morning (6:00 a.m.) until evening (6:00 p.m.) and ants in evening hours of 5.30 - 7.30 pm (Fig.3g). Extra floral nectaries (pedicellarnectaries) of *B. orellana* are regularly visited by ants and may affect seed set. However, the flowers themselves have no floral nectaries. Thus, the pollinators visit the flowers only to collect pollen. A variety of insect visit *Terminalia arjuna* during the flowering period, but bumble bee is found to be the most efficient pollinator (Chauhan and Sigh, 2001). The most frequent pollinators are species in the bee tribe Euglossini (Bentley 1977). Under the natural conditions, an average of 43 % fruits attains maturity with a range of variation (27 % to 57 %) among different variants of annatto (Joseph *et al.* 2012). *Bombus* bees, which were registered in our field observations, are considered by Abak *et al.* (2000) to be efficient pollinator agents due to their intense activity and increased vibration. These authors showed that *Bombus* pollination increased fruit size and the number of seeds per fruit in eggplant (*Solanum melongena*). Bentley (1977) showed that ants visiting extra floral nectaries of *B. orellana* increased fruit production, probably by supplying protection for floral tissues against herbivores.

Pollen productivity depends on anther length, pollen grain size and mode of anther dehiscence. In all the three CPTs, tricolporate pollen was observed (Fig. 3e). Among the four stains used, the stain I-KI showed 95 % pollen viability (Fig. 3i) and it is found that the viability of the pollen grains decreased after 4 h from anther dehiscence. Viability of the pollen at different times showed a significant decline from four hours after dehiscence of pollen. Nisha Joseph *et al.*, 2012 reported that pollen viability was evaluated using the fluorochromatic (FCR) test and the time period in which viability of pollen is retained is crucial and varies from species to species and the time period in which viability of pollen is retained is crucial and varies from species to species.

Stigma receptivity is known to be an essential stage in flower maturation and it may significantly influence the rate of self-pollination and pollination success at different stages in the flower life cycle (Dafni and Motte, 1998). Stigma receptivity in *B. orellana* in Costa Rica has been reported to occur over a period of 4 to 6 hour (Rodriguez and Enriquez, 1983) but in the Yucatan Peninsula to occur consistently from 0800 or 0900 to 1200 hours (Rivera Madrid *et al.*, 2006). In the present study, stigma were examined periodically using the stigmatic peroxidase activity test, and eight hours after anthesis, stigma showed rapid bubbling of oxygen from the added H<sub>2</sub>O<sub>2</sub>. Thus it can be assumed that the stigmas have maximum receptivity by 1200 hours.





**Fig. 3. Floral, leaf and seed characters and its variation of annatto**

a. Inflorescence (Compound Raceme); b. Anther and stigma arrangement of a *Bixa* flower; c. anther with viable pollen grains; d. Flower during anthesis; e. Seed setting on selfing; f. Open Pollination g. Insect visit during anthesis; h. Seeds; i. Pollen viability (I-KI); j. Seeds of TN CPT; k. Seeds of Kerala CPT; l. Seeds of Karnataka CPT; m. Variation in Leaf; n. Colour variation in Bristle

In *Bixa*, stigmas are fully surrounded by anthers in such a way that self-pollination is ensured. Pollen is shed directly on the stigma when the anthers open enabling self-pollination. However the slightly elevated position of the stigmatic surface leads to cross pollination (Fig. 3b) and due to the high amount of phenotypic variability observed in the characters, however, in crops considered to be cross-pollinated, some degree of self-pollination usually occurs. In the this study, the inflorescence was selfed and artificially pollinated to find out the mode of pollination by covering their flower buds in all the three CPTs and also they pollinated with other plant pollen. From the experiment, it can be concluded that *Bixa* is a self and cross pollinated crop based on fruit set (Fig. 3ef). The arrangement of anther and stigma are depicted in the fig.3b and pollen dehiscence viewed (Fig. 3c) at the time of peak flowering. Pranesh *et al.* (2010) studied the mode of reproduction of *Jatropha curcas* and based on the treatments of emasculation, bagging and artificial pollination, which indicated that *Jatropha* could facilitate sexual system geitonogamy up to 75.6% fruit sets indicates self-compatibility and xenogamy up to 82.33 per cent fruit set. Nisha Joseph *et al.* (2012) studied the reproductive characterization in annatto and reported that based on fruit setting, this plant can have both self and cross pollination and Valdez-Ojeda *et al.* (2010) also in tune with the reports that annatto can accept both self- and cross-pollination.

The characters *viz.*, floral, biometrical and yield contributing characters (54 nos.) were observed to characterize the three candidate plus trees (CPTs) of *Bixa viz.*, Kerala, Karnataka and Tamil Nadu (dark red fruited, red fruited and green fruited plant respectively) and these CPTs showed lot of phenotypic variations in the plant stature, leaf size, flower colour, bristle size, fruit colour, fruit shape and seed size. The morphological features of the plants used in the breeding experiments are illustrated in figure 2. Morphological characters, of the parent plants selected for the controlled breeding experiments were analyzed. Mean performance in terms of growth characters and yield traits showed significant differences among the three source plants (Table 1). The use of leaf morphometrics in *Salix* was investigated by Aravanopoulos (2010) at three genetic entry levels and reported that the Leaf and stipule shape parameters emerged as most important variables. Separation at the family level was partially achieved in a tri-dimensional ordination of the major axes in both principal and discriminant space.

The minimum height was observed in Karnataka CPT (1.35m) and it varied from 1.52 to 1.84 m with an average of 1.64 m in Tamil Nadu CPT and 1.25 to 1.48 m with an average 1.36 m in Kerala CPT. The number of tertiary branches and the number of inflorescence per tertiary branch gives approximate number of inflorescence in the tree. Since the tertiary braches are found to be flowering

**Table 1. Growth and yield characteristics of three CPTs of *Bixa orellana***

GENOTYPES/ CHARACTER	HT	CD	PB	SB	IL	R/IN	LL	LW	PED LEN	F/IN	FR/IN	FS %	FL	FW	SEED/ FR	100 SWT	YLD/PL (gm)
GREEN FRUITED CPT	1.59	5.58	7.40	46.20	8.66	8.60	15.78	10.42	1.18	34.20	13.00	38.00	3.80*	2.76	43.40	3.04	43.38
RED FRUITED CPT	1.31	4.78	10.20	46.60	9.62	10.80	16.38	10.90	1.54*	48.00	22.20	52.32*	3.58	3.48*	48.00*	<b>3.42*</b>	<b>113.12*</b>
DARK RED FRUITED CPT	1.36	4.85	9.80	34.80	9.22	10.60	17.92	11.88	1.30	62.60*	33.80*	54.31*	3.76*	2.26	50.20*	3.21	76.92
MEAN	1.42	5.07	9.13	42.53	9.17	10.00	16.69	11.07	1.34	48.27	23.00	48.21	3.71	2.83	46.20	3.22	77.81
SEd	0.08	0.75	0.81	2.95	1.72	0.75	1.08	0.92	0.13	3.03	1.68	3.64	0.31	0.16	1.45	0.06	5.24
CD	0.18	1.73	1.86	6.91	3.96	1.73	2.49	2.12	0.29	6.99	3.89	8.03	0.71	0.36	3.35	0.14	12.08

HT: Plant height; CD: Collar diameter; PB: No. of primary branches/plant; SB: No. of secondary branches/plant; IL: Inflorescence length; R/IN: No. of rachis/inflorescence; LL: Leaf length; LW: Leaf width; PED LEN: Peduncle length; F/IN: No. flowers/inflorescence; FR/IN: No. of fruits/inflorescence; FS%: Fruit setting percentage; FL: Fruit length; FW: Fruit width; SEED/FR: No. of seeds/fruit; 100 SWT: 100 seed weight; YLD/PL: Single plant yield; CPT: Candidate plus tree

shoots. Variation in leaf size, colour and bristle colour also observed in this study (Fig. 3m and n). The length of the inflorescence varied from 6.8 to 13.4 cm with an average of 8.8 cm (TN), 8.6 cm (KL) and 9.4 cm (KA). The high length of inflorescence gives more number of flower productions. This similar observation made by *Wunnbea dioica* by Vaughton and Ramsey (2012). The inflorescence length is also a reason for high seed production (Barret *et al.*, 1999). The number of fruits produced is based

on the length of inflorescence, number of rachis per inflorescence. The number of flowers per inflorescence was found to be 34.20 (TN), 48.00 (KA), 62.60 (KL). The number of rachis per inflorescence was 8.60 (TN), 10.80 (KA), 10.60 (KL). The number of branches that directly emerges from the main branch were 7.40 (TN), 10.20 (KA), 9.80 (KL). The number of branches that emerges from the primary branch were 46.20 (TN), 46.60 (KA), 34.80 (KL).

The fruit colour of *Bixa* for three CPTs were visually assessed and was found to be light red colour (green fruited) in Tamil Nadu and moderate red colour in Karnataka and dark colour in Kerala (Fig 3 j,k,l). Akshatha *et al.*, 2011 have studied on morphological diversity in *Bixa orellana* L. and variations in annatto pigment yield and observed ovate red, conical greenish red and hemispherical green fruiting varieties. The average length of the fruit varies from 3.4 cm (KL) to 3.8 cm (TN). Fruit maturity takes place 80 – 95 days counting from the day of pollination to fruit dehiscence. The shape of the fruit resembles the type of rounded oblong pyriform in all the three and it matured around 90 days. On selfing, the maximum fruit set (85%) and succeeding maturation of fruits (72%) was recorded with the red-fruited variety. The number of seeds per fruit (33.80) was found to be high in the dark red fruited variant, whereas the number of capsules per bunch was found on par in the brown variant. Maximum fruit setting percentage and 100 seed weight (3.42 g) was observed in red fruited variety (Karnataka) followed by dark red fruited variety. These yield contributing characters have a direct association with the yield potential of the plants, and hence it can be used as parent plants for further breeding program to produce recombination of ideal traits. The qualitative characters analyzed will be helpful in the easy identification of plants. Maximum seed production was recorded by the red fruited (collected from Karnataka) (113.12 g) plant followed by the dark red fruited plant (Kerala) and the green fruited variant (TN). Rivera-Madrid *et al.*, 2006 reported that only 40% of annatto fruits attain maturity and the seed output is around 50% under natural conditions.

The results of the present findings provide a step towards definite genetic improvement of *bixa* and the standardization of controlled breeding strategies for annatto will be helpful in further breeding programme for raising superior cultivars appropriate for the different agro-climatic conditions of Tamil Nadu. For evolving genetically superior *Bixa* plants with desired characters including a high bixin content, seed output and synchronous flowering, international efforts and exchange of germplasm are needed (Joseph *et al.*, 2012).

The regulation of controlled breeding strategies for *Bixa* in the Indian subcontinent will be helpful in further breeding and genetic studies for raising the superior genotypes suited to the different environmental condition of Tamil Nadu.

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