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Morphological characterization of floral traits to predict ideal stage for haploid production in bell pepper

K. P. Sahana, Khushboo Singh, Arpita Srivastava, Anil Khar, Neelu Jain[1], P.K. Jain[2], D. Vijay[3], Hemlata Bharti[4], A. K. Singh[4] and Manisha Mangal[*]

Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi 110012, Delhi, India.

ABSTRACT

Double haploid (DH) breeding helps shorten the breeding cycle and fastens the release of homozygous inbred lines with superior and desirable traits. *In vitro* microspore culture is the fastest approach for producing haploid plantlets. The critical parameter for the success of haploid production in any crop species is the stage of microspore or pollens at the time of culture as it decides the fate of cultured anthers/pollens. Therefore an easy, precise, fast, and reliable criterion to identify flower buds carrying microspores or pollen at particular stages is essential. In the present investigation, we developed the easiest and most accurate criterion to correlate visible, measurable traits of bud and anther development with individual stages of microsporogenesis in two sweet pepper F1 hybrids (Orobelle and Bomby). Anthers containing microspores at the late uninucleate to the early binucleate stage are optimal for the induction of androgenesis in a wide range of crop species. Anther containing uninucleate pollens were observed in flower buds with an average size of 5.39 mm in Orobelle and 4.8 mm in Bomby, an average anther length of 2.75 mm in Orobelle, and 2.71 mm in Bomby, and corolla was slightly longer than that of the calyx in both the hybrids. Anthers had no purple pigmentation in Orobelle and slight /no pigmentation at the top of the anther sac end in Bomby.

Keywords: Sweet pepper, anther culture, haploid production

INTRODUCTION

Sweet pepper (*Capsicum annuum* L.) is one of the popular commercial and export oriented crop grown worldwide for vegetable, spice, ornamental, medicinal and lachrymatory uses and is an important dietary source of vitamins A, B-complex, C, E and minerals like molybdenum, manganese, folate, potassium and thiamine (Bosland *et al.,* 5). It originated in the New World tropics and sub tropics. Mexico is the centre of diversity for *C. annuum.* The genus Capsicum contains over 30 species, five of which are domesticated (C*. annuum, C. frutescens, C. chinense, C. baccatum,* and *C. pubescens*) and are primarily grown for consumption. The genetic improvement of sweet pepper has been limited to conventional breeding tools which essentially involve self-pollination over generations to obtain homozygous true breeding lines containing genes related to agronomic and resistance traits. However, obtaining pure homozygous lines by conventional method is time consuming and labour intensive since it requires at least six to seven generations of self-fertilization. Alternatively production of haploids and doubled haploid plants through biotechnological approaches is useful since it takes one generation to develop pure lines which in turn reduces time and production costs.

Apart from breeding programmes aiming for improved cultivars, DH also facilitates the selection of recessive mutants and identification of traits governed by recessive genes. It also provides valuable material for mapping, genetic and cytogenetic analysis. Studies on the anther culture of the genus *Capsicum* have been initiated in other parts of the world by Wang *et al.* (15), whereas George and Narayanaswamy (8) reported microspore embryogenesis in pepper and subsequently androgenesis induction in European varieties was reported by Novak (12). Dumas de Vaulx *et al*. (7) established the basis for a general, reliable method for anther culture in *Capsicum* spp. However, pepper is considered as a recalcitrant species owing to its low (0-10 percent) frequency of the development of androgenic embryos (Seguı´-Simarro *et al.,* 14).

Successful production of androgenic haploid plants and DH depends on several factors such as genotype, prior treatment of anther/ bud, development stage of mircospore. Many researchers reported that, anthers containing microspores at late uninucleate to early binucleate stage are optimal for induction of

*Corresponding author: manishamangal@rediffmail.com

[1]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, India

[2]ICAR-National Institute for Plant Biotechnology, New Delhi, India

[3]Division of Seed Science Technology, ICAR-Indian Agricultural Research Institute, New Delhi, India

[4]Centre for Protected Cultivation Technology, ICAR-Indian Agricultural Research Institute, New Delhi, India

androgenesis in wide range of crop species (Mishra *et al*.,11; Ali *et al*., 2). Similarly in pepper also uninucleate microspore and young binucleate stage microspores have been found to be ideal for haploid generation in previous findings (Lantos *et al.,* 9; Barroso *et al*., 3). Morphological changes in bud and anthers accompanied by changes in microspore and pollen development could be exploited for identification of ideal stage needed for successful production of haploid plants. Sizes of flower bud and anther, calyx to corolla ratio, anthocyanin pigmentation, pectin esterification level etc. have been used as marker for identification of microspore development stage (ParraVega *et al.*, 13; Mangal and Srivasatava, 10) in earlier studies. The present study aimed to establish correlation between bud and anther parameters with stage of microsporogenesis/micrgametogenesis in bell pepper (*Capsicum annuum* L.) hybrids Orobelle and Bomby.

MATERIALS AND METHODS

The pepper seedlings (*Capsicum annuum* L.) of two commercial hybrids, Orobelle and Bomby, were transplanted at the National Phytotron facility, IARI-New Delhi, in the month of October 2019, and maintained under controlled temperature conditions of 25±2°C. Buds for experimentation were taken from the first flush of flowering in the month of December.

Flower buds of pepper cultivar Orobelle ranging from 5 to 8mm in length and of Bomby ranging from 4 to 7.5mm were used for the study. Buds, calyx lengths and anther sizes were measured with the help of Magcam DC5 software under stereo microscope (Olympus SZX7, DF PLAPO1.25 X , Tokyo, Japan). The length was estimated for buds from the point of insertion of the pedicel to the tip of the bud and for anthers, from the point of insertion of the filament to the tip of the anther (apical end) (Parra Vega *et al.*, 13). Three stages of bud development were studied (Table1). The unopened flower buds were collected randomly from different developmental stages during morning hours. For each of the bud stages used, three replications were used and each replication comprised of three buds. An average measurement of nine buds is presented in Table1. The observed bud characteristics included (i) whether the bud was open or closed; (ii) the length of the corolla and the calyx in the buds. Two anthers taken from each bud, were observed for pigmentation and then used for identification of microspore/pollen stage. Observations from 6 anthers (two anthers per bud; three replications of 1 bud each) were used to estimate the level of anther pigmentation as well as to estimate the stage of microspore development. In order to obtain pollens, anthers were crushed with the help of a pair of forceps on a microscopic slide. The pollen grains so obtained were washed with 1 X PBS buffer for 1-2 times and excess solution was removed. These pollen grains were treated with 300nM fluorescent DNA stain (DAPI-4′,6-diamidino-2-phenylindole)for a period of 5minutes under dark conditions followed by removal of excess stain and washing with PBS buffer.

Following the DAPI staining method, the microscopic slides were prepared and observed under Leica inverted fluorescence microscope (model DMi8, LAS X software, DHR Holding India Pvt. Ltd., New Delhi-India) at a total magnification of 400X with 40X objective and 10X eyepiece to determine developmental stages of microspores.

RESULTS AND DISCUSSION

Pepper is considered as recalcitrant crop for *in vitro* androgenesis process. Characteristics of flower buds and anthers, that can give us an indication of the stage of microspore inside the anther that is most conducive to the androgenic doubled haploid production, is very important for successful haploid regeneration. The stage of microspore development in anther can be correlated with shape, size and colour of flower bud and anther.

The findings of present investigation pertaining to the size and morphological characteristics of the buds, anthers and stage of microsporogenesis have been depicted in Table 1 as well as in Fig. 1 & 2.The microspore stages within the anthers obtained from different sizes of buds were determined through microscopic analysis and delineated in Fig. 1.

Anthers taken from unopened flower bud (developmental stage I) of average length 5.39 mm in Orobelle and 4.8 mm in Bomby contained uninucleate microspore with outsized cytoplasmatic cavity and having nucleus pushed towards the exine. This stage has been reported to be the foremost contributive stage for successful androgenesis in pepper. In bud developmental stage II, microspores showed initial spore cellular division and young bicellular pollens were observed while mature pollens were observed at developmental stage III. Our results showed that buds at developmental stage I contained uninucleated microspores, which is the best suitable development stage for inducing androgenesis in pepper. The morphological markers discovered for this stage in our studies were found to be average anther length of 2.75 mm and average flower bud length of 5.39 mm for in Orobelle whereas these parameters were 2.71mm and 4.8mm respectively

Table 1. Characteristics of buds and anthers and their correlation with microspore development in Bell pepper hybrids Orobelle and Bomby

Stage	Bud measurements						Anther measurements				Characterization of buds	Characterization of anther	Stage of microsporogenesis
	Orobelle			Bomby			Orobelle		Bomby				
	Length[1]	Diameter[1]	Corolla –calyx length (mm)	Length[1]	Diameter[1]	Corolla –calyx length (mm)	Length[2]	Diameter[2]	Length[2]	Diameter[2]			
I	5.39	5.66	1.89	4.8	5.52	1.87	2.75	1.40	2.71	1.33	The corolla of buds was slightly longer than that of the calyx. The difference in Corolla-calyx length was 1.89 mm in Orobelle and 1.87 mm in Bomby	The anthers are pale yellow to green with no pigmentation on anther sac ends in Orobelle but slight and sometimes no pigmentation in Bomby	Microspores at uninucleate stage or Vacuolate (mature microspores with thick exine walls)
II	6.01	6.23	1.96	5.79	6.41	2.34	2.89	1.46	3.11	1.37	The corolla of buds was longer than that of the stage I. the difference was found to be 1.96 mm and 2.34 mm in Orobelle and Bomby respectively	The anthers have purple tinge on 1/3rd of the anther length and rest of the portion is green in Orobelle while 1/6th of the anther length has purple tinge in Bomby	Microspores at 1st pollen mitosis stages, young bicellular pollen
III	6.85	7.12	3.32	6.78	7.27	3.40	2.97	1.50	3.31	1.48	The calyx covered only half of the corolla length. The difference between corolla and calyx was 3.32 mm in Orobelle and 3.40 mm in Bomby	2/3rd of the anthers is purple and rest portion is green in Orobelle whereas in case of Bomby 1/3rd of the anther length has purple pigmentation	Mature pollen

[1]Average of 3 buds in mm [2]Average of 6 anthers in mm

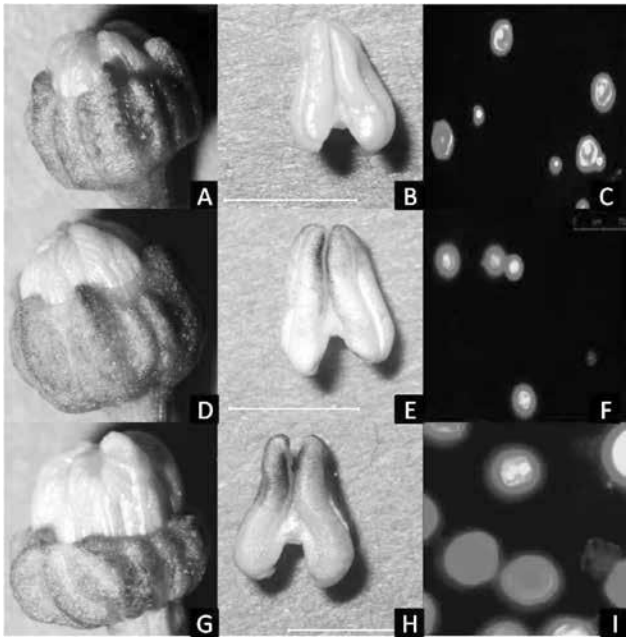

Fig. 1. Morphological and microscopic features of bud,anther and microspore in Orobelle

(A-C : Represents flower bud, anther and microspore of Stage-I; D-F : represents flower bud, anther and microspore of stage-II and G-I : flower bud, anther and microspore of stage III)
*Scale bar represents a length of 3mm

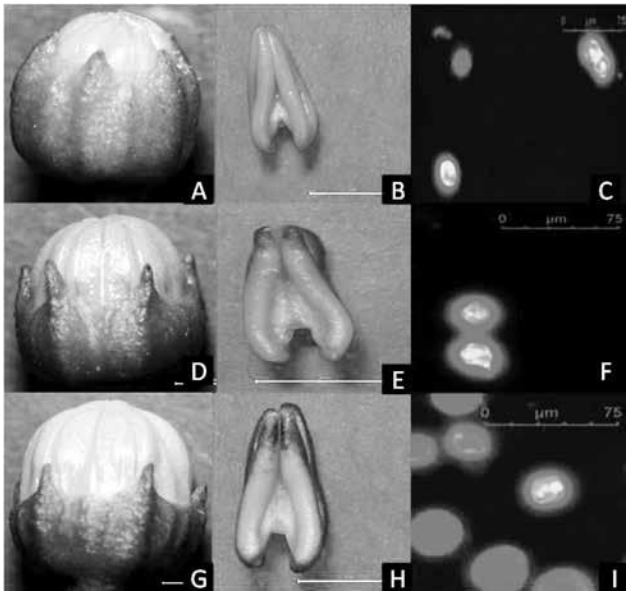

Fig. 2. Morphological and microscopic features of bud,anther and microspore in Bomby

(A-C : Represents flower bud, anther and microspore of Stage-I; D-F : represents flower bud, anther and microspore of stage-II and G-I : flower bud, anther and microspore of stage III)
*Scale bar represents a length of 3mm

in Bomby. With respect to calyx corolla ratio and anther pigmentation our studies revealed that at this developmental stage I, corolla was slightly longer than that of the calyx and calyx covered almost 90% of corolla and no purple pigmentation was observed at the top end on the surface of the green anthers in Orobelle and slight /sometimes no pigmentation at anther sac end in Bomby. Further, it was also observed that once the buds and anthers were chosen based on these parameters they contained nearly 90% microspores in the uninucleate stage.

Previous studies aiming androgenesis and DH development in pepper also reported similar observations. Barroso *et al.* (3) had defined the size and morphology of the buds and anthers for predicting microspore stages. Parra Vega *et al*. (13) had also used anther/bud length, anther pigmentation and calyx/ corolla ratio as predictors of stage of development of microspore. Contrastingly, Ciner and Tipirdamaz (6) showed that the stage of flower bud when corolla and calyx are of identical length is the most suitable stage for initiation of embryogenesis in pepper while Novak (12) reported that buds ranging from 2.6 to 5.0 mm in size contains anthers with uninucleated microspores and provided good results with respect to pepper haploid development. It has also been reported that, microspore developmental stage of the anther is genotype dependent. We also observed slight changes in different morphological parameters in the two genotypes studied under present investigations which is in line with previous findings involving wide range of crop species which suggest that the genotype is an important factor in determining successful haploid induction (Başay *et al.,* 4; Adhikari and Kang,1). The present study concluded that the combined use of calyx/corolla quantitative relation and reproductive structure pigmentation could give simple, quick and reliable identification of buds and anthers containing uninucleate or early binucleate stage microspore i.e., the most appropriate stage for anther culture in pepper.

AUTHORS' CONTRIBUTION

Conceptualization (MM, NJ, PKJ); Designing of the experiments (MM, AS, AK); Contribution of experimental materials (HB, AKS); Execution of field/lab experiments and data collection (SKP, DV); Analysis of data and interpretation (MM, SKP); Preparation of the manuscript (MM, SKP, AK).

DECLARATION

The authors declare that they have no conflict of interest.

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