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FULL-LENGTH RESEARCH ARTICLE

Development and Maintenance of Tropical Gynoecious Inbred Lines in Cucumber (*Cucumis sativus*) and Validation by DNA Markers

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Abstract Gynoecious parthenocarpic hybrids in cucumber (*Cucumis sativus* L.) have great importance for its successful cultivation under protected conditions to enhance productivity and quality. Work was undertaken to develop and maintain the gynoecious inbred lines from the gynoecious parthenocarpic cucumber hybrids (Silyon hybrid, Pickling cucumber-1 (PC-1), Pickling cucumber-2 (PC-2) and Pune cucumber hybrid) at ICAR–Indian Institute of Horticultural Research, Bengaluru. Four slicing cucumber lines, namely IIHR-434, IIHR-435, IIHR-436 and IIHR-437, were raised to develop segregating populations. The individual plant selections were made in F_2 population and through the pedigree method. These lines were forwarded to F_4 generation based on the gynoecy sex expression, and mean performance of all four gynoecious lines was recorded. The F_4 populations were validated at phenotypic as well as through SSR markers linked to gynoecious trait. The SSR-02021 and SSR-18718 genotypic data showed that, most of the plants are gynoecious in all four advance gynoecious lines. These inbred lines are further utilized to develop gynoecious parthenocarpic cucumber F_1 hybrids.

Keywords Gibberellic acid · Gynoecious · Monoecious · Silver nitrate · Hybrids · Parthenocarpy

Introduction

Cucumber (*Cucumis sativus* L.) belongs to the Cucurbitaceae family, which comprises of 118 genera and 825 species [13]. It is thought to be one of the oldest vegetable crops and cultivated for over 3000 years in India [9]. Cucumber occupies 2.6 million hectares in the world cultivated area with an annual production of 44.3 million tonnes. In India, it is grown in an area of 77,000 hectares and produces 1.2 million tons [25]. The yield of cucumber depends on the number of female flowers produced per plant. Hence, the female sex expression (gynoecy) plays an important role in cucumber breeding program because the commercial F_1 hybrid seeds were generated by crossing two different unisexual cucumber breeding lines. Therefore, selection of gynoecious cucumber lines for maternal parents is highly crucial process in cucumber breeding. Traditionally, the selection of gynoecious cucumber lines is made based on the observation of flower sex expression in breeding lines. The traditional method has several limitations such as selection accuracy, stability, early identification and maintenance of gynoeciousness. The application of molecular marker (SSR marker)-aided selection of gynoecy can enable breeders to select gynoecious advance breeding lines on the basis of a simple cost-effective DNA assay without undertaking extensive phenotypic evaluation. These markers must be reliable, repeatable and closely linked to reduce the probability of recombination. These genetic markers may be exploited to develop an efficient MAS strategy for breeding gynoecious in cucumber cultivars [35].

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Gynoecious sex form was initially spotted out as a chance seedling segregated from a Korean gynomonoecious introduction 'Shogoin' (PI 220860), and gynoecious lines were developed [27]. A series of gynoecious lines were developed in South Carolina [2, 3] and Cornell [23], USA and Netherlands [16]. The homozygous gynoecious sex forms as parents or F_1 hybrids (gynoecious x gynoecious) were stable under moderate regimes of temperature and photoperiodic conditions, but where the temperature exceeded beyond 30 °C, the stability of gynoecious sex expression is affected [7, 21]. The gynoecious lines were grown in tropical countries, like India under high temperature and long photoperiodic conditions, which drastically are affected its stability; thus, the gynoecy in cucumber did not receive much attention in the tropical countries. More and Seshadri [22] attempted to transfer gynoecy into tropical varieties of cucumber with four stable tropical gynoecious lines. The gynoecy is governed by single dominant allele, often bearing a high proportion of female flowers, resulting in earliness, good yield and give many fruits in a single harvest. In India, the F_1 hybrid Pusa Sanyog has been released by IARI, Katrain campus [12] by crossing gynoecious line, isolated from a Japanese variety Kaga Aomoga Fushinavi with Green Long of Naples (Italian variety).

In addition to climatic influence, sexual differentiation of floral primordia depends on hormonal balance in primordial tissue and also can be altered by exogenous application of hormones. Gibberellins favor masculinization, auxin, ethylene and cytokinin enhance feminization [20]. Reduction of ethylene level in tissues causes formation of staminate flowers in place of pistillate ones [6]. Peterson and Adlher [27] reported that 1500–2000 ppm GA_3 promoted male flowers in gynoecious cucumber lines. Silver nitrate ($AgNo_3$) at 200–300 ppm is effective for male flower induction [4, 10, 21]. Several researchers have worked on sex expression of cucumbers and reported that it was genetically determined but could be modified by application of growth substance and also environmental factors [15, 17, 19]. Considering the above factors, many combinations of hybrid seed production techniques have been proposed and recommended using gynoecious parents. Despite of all efforts, variation in sex expression of commercial hybrids is still a problem in cucumber cultivation [19]. Therefore, the experiment was designed to develop and stabilize the gynoecious character in cucumber and maintain the stable gynoecious inbred lines with marker validation to gynoecy.

Materials and Methods

Sex Expression in Original Population (F_1) and Development of Gynoecious Lines

The four gynoecious parthenocarpic cucumber hybrids, namely Silyon hybrid (Collected from Rijkzwaan Seeds pvt. Netherland), Pickling cucumber-1 (PC-1), Pickling cucumber-2 (PC-2) and Pune cucumber hybrid, (Collected from ICAR-Indian Institute of Horticultural Research, Bengaluru), were initially raised in plastic trays and later transplanted in the polyhouse of the Division of Vegetable Crops, ICAR- Indian Institute of Horticultural Research, Bengaluru, India, on December 15th, 2015 for a preliminary study. Plants were examined for pistillate expression at first five nodes. In individual plant, the sex expression percentage was recorded [32, 33]. The best performing gynoecious plants were treated with 300 ppm GA_3 to induce staminate flowers to develop F_2 and further segregating populations. In F_2 population, the individual plant selections were made and through the pedigree method, they were forwarded to F_4 generation based on the gynoecy sex expression, and mean performance of all four gynoecious lines was recorded. The selection of the plants and statistical analysis was done through Chi-square contingency test [34].

Silver Nitrate and Gibberellic Acid Concentrations for the Maintenance of Gynoecious Lines

The experiment was conducted with gynoecious parthenocarpic cucumber line 'IIHR-434' under polyhouse condition during Rabi 2016. Four treatments were imposed under randomized block design with four replications, and observation was recorded on randomly selected five plants in each plot. The two concentrations of each chemical, viz., $AgNo_3$ (silver nitrate) 1.2 mM and 2.4 mM and GA_3 (gibberellic acid) 1.3 mM and 2.0 mM, were applied in four times. The first application was started at 10^{th} day after planting and succeeding application at 15-day interval. All chemical solutions were prepared with deionized water and sprayed on plants. Data were collected on five randomly selected plants per replication for days to first male flower, number of male flowers, pedicel length, node for first male flowering, number of female flowers, total number of flower and percentage of altered flowers. Data were transformed in arc sine transformation for statistical analysis using Statistical Analysis System (SAS) software (SAS, package available at ICAR-IIHR, Bengaluru).

Mean Performance of Gynoecious Cucumber Lines (F_4) for Quantitative Characters

The experimental genotypes comprised of four gynoecious of cucumber viz., IIHR-434, IIHR-435, IIHR-436, IIHR-43. The observations viz., days to first female flower, nodes to first female flower, fruit length (cm), fruit girth (cm), fruit weight (gm), number of fruits per plant, yield per plant (kg) were recorded on the randomly selected five plants in randomized complete-block design (RCBD) with three replications. The data collected on the quantitative characters were subjected to Fisher's method of analysis of variance (ANOVA) as per the methods outlined by Panse and Sukhatme [26]. The critical difference (CD) calculated wherever the 'F' test found significant. The data were analyzed in Statistical Analysis System (SAS) and presented at the five percent level of significance ($P < 0.05$).

Validation of DNA Markers for Gynoecious Character in Cucumber

Four gynoecious lines were used for marker validation, which belongs to the F_4 families, namely IIHR-434, IIHR-435, IIHR-436 and IIHR-437. These lines were developed by breaking gynoecious commercial F_1 hybrids of slicing cucumber, derived through selfing after modifying into staminate sex form through hormonal treatment. Twelve reported SSR primers for gynoecy in cucumber were used in the study (Table 1). DNA was extracted from young cucumber leaves using DNeasy plant mini kit. Primers were screened with individual gynoecious plants and compared with monoecious cucumber variety Swarna Agethi for amplicon size polymorphism. Genomic DNA (20 ng) was amplified in a reaction mixture with programmed at 94 °C for 5 min 30 cycles of 94 °C for 15 s, 55.5 °C for 30 s and 72 °C for 30 s and a final extension for 5 min. at 72 °C. The amplified products were electrophoresed through 3% agarose gel in TAE buffer (separating very tiny fragments of DNA with good resolution for small of 0.1–1 kb fragments) and photographed on the gel documentation system.

Results and Discussion

Sex Expression in Original Population and Development of Gynoecious Lines

The four gynoecious parthenocarpic cucumber hybrids which showed stable gynoecious in the original population (F_1) were taken for the development of gynoecious inbred lines. There was a significant increase in gynoecious plant percentage in F_4 generation all the cucumber lines (Table 2). The highest percentages of gynoecy (92%) observed in IIHR-434 followed by IIHR-436 (90%), IIHR-437 (89%) and IIHR-435 (88.5%) and mean separation in

Table 1 List of primers with sequence and base pair difference used for validating the cucumber F_4 populations for gynoecious character

Primers	Gynoecious (Base pairs)	Monoecious (Base pairs)	Forward and reverse primer sequence
CSWCT25	182	165	AAAGAAATTAAGTCAATCAAACCG
			CCCACCAATAGTAAAATTATACAT
SSR18956	178	192	CGTATGTACGACAAAATGTGAACAG
			TCGAAACCTCAATACTTCTACCAA
Cs-BCAT	160	216	CATTGTGTGAATGAAGACAAG CTTCAACGCAAAACCTTCATC
Cs FEMALE1	163	197	TGGAGATAAAGCGTAAGGGAA
			CCTCCAACGTCATAGAGTAAA
Cs-FEMALE-4	220	204	CGATCAGATATAACTGCAGCAGT TAATAGTCGCTGCCAAGTAAAGC
Cs-FEMALE-7	110	122	TGGTTTGGTTTTTAGGGGAGA CCCCACGTTACAAAATAGAAG
SSR-02021	150	190	TAAACATGGCTTCCTCCTCC
			CTCTCTTTTCTCACACCCACAG
SSR-15818	220	200	GGACATGTCAACTCCCCTGT GCCTCTAGCCTGAAAGACCA
SSR-15516	200	220	TAAAACACCCAATCGCCAAT GTGGACGAGAGGGATGGATA
CSWCT-17	190	130	TTGAATTATGGGTTCATTTTT GACAATGATAAACTTCCCTGA
SSR-17481	190	150	GAGGTGGCAGCTGAAAAGAG TTCATTCGAATAACCTGCCTC
SSR-18718	180	150	TGAAGCAAAGTAACCCCACA CACAAATGGATTACAGAGCGAA

Table 2 Percentage of gynoecious plants in different generations of four cucumber cultivars

Selfed generations	IIHR-434-3-8-5	IIHR-435-6-18-8	IIHR-436-2-7-13	IIHR-437-4-19-9
(F_1)	100[a]	100[a]	100[a]	100[a]
(F_2)	77	64	74	72
(F_3)	85	82	85	82.5
(F_4)	92	89.5	90	89
Observed Chi-square at 5%	0.15	0.05	0.05	0.19

[a]Monoecious

column by 'Chi square contingency' test indicate differences between generations in gynoecious plants at 95% confidence (Fig. 1). Percentage of gynoecious plants in F_4 generation were found to be significantly increased at 95% confidence. All F_1 populations are complete gynoecious plants because F_1 might have been developed from gynoecious parents and also, it is controlled by a single dominant gene. Selfed progenies of gynoecious plants followed by plant-to-row selection system had increased the percentage of gynoecious plants over the generations in all populations. It is obvious that by inbreeding and plant-to-row selection, the traits become fixed and the progeny or line approach increases uniformity [1]. The results obtained in the present investigation are in agreement with those reported by Porchazkova [28], and Chen et al. [8] found better performance of tropical gynoecious parthenocarpic lines.

Maintenance of Gynoecious Lines

Silver nitrate and gibberellic acid sprays influenced the flowering characters (Table 3). The plants showed an increased number of staminate flowers (12.17) at earlier nodes (11.75) and the appearance of first flower in 47 days after sowing by spraying with gibberellic acid at 1.3 mM. The longest pedicel of staminate flowers was due to treatment with gibberellic acid at 2 mM followed by gibberellic acid at 1.3 mM. Increased pedicel and vine length might be due to gibberellic acid treatment, which is in accordance with the results of Singh and Choudhary [31] in watermelon, cucumber and bottle gourd. The enhanced vegetative growth could be due to increased plasticity of the cell wall followed by hydrolysis of starch to sugars lowering cell water potential resulting in entry of water into the cell causing elongation [29]. The osmotic-driven responses due to gibberellins increase photosynthetic

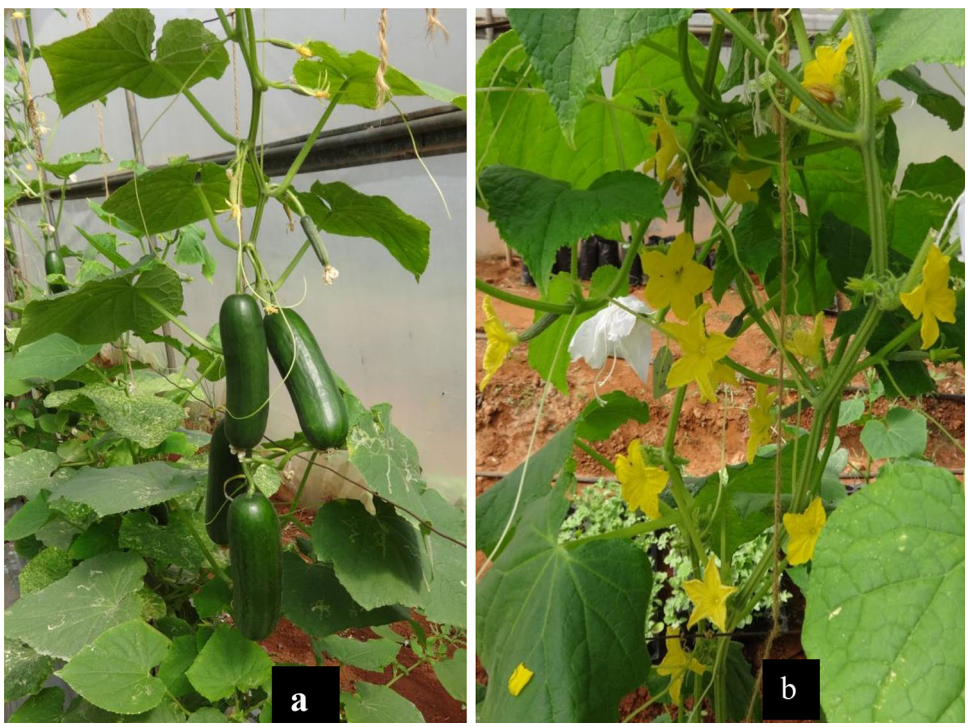


Fig. 1 Gynoecious (a) and monoecious (b) plant in F_4 population of cucumber inbred line IIHR-437

Table 3 Effect of silver nitrate and GA_3 on gynoecious cucumber line (IIHR-434) for flowering characters

Treatments	Days to 1st male flower appearance	Number of male flowers	Node to 1st male flower appearance	Pedicel length (cm)	Number of female flowers	Total Number of flowers	Total altered flowers (%)
$AgNo_3$ @1.2 mM	55.5	3.1	20.25	3.40	28.1	31	10
$AgNo_3$ @2.4 mM	50	5.6	11.5	4.40	22.4	28	20
GA_3 @ 1.3 mM	47	12.17	11.75	2.10	21.8	34	35.80
GA_3 @ 2 mM	48.25	8.73	17.75	4.20	21.27	30	29.10
SE ± m	0.728	0.094	1.081	0.038	0.148	0.225	2.321
CD (5%)	2.361	0.305	3.507	0.123	0.480	0.730	7.531

activity, accelerates translocation and efficiency of utilizing photosynthetic products resulting in cell elongation and rapid cell division. Treatment of gynoecious plants with silver nitrate at 1.2 and 2.4 mM did not respond well for staminate flowers production. The plants sprayed with gibberellic acid at 1.3 mM recorded the highest number of female flowers altered into male flowers (35.80%), followed by 29.10% in GA_3 2 mM treated plant. Thus, it would be interesting to determine if the gibberellins modify sex expression through auxin metabolism or more directly by altering the rate of development of staminate primordial in cucumber [5].

Mean Performance of Gynoecious Cucumber Lines (F_4) for Flowering and Yield Characters

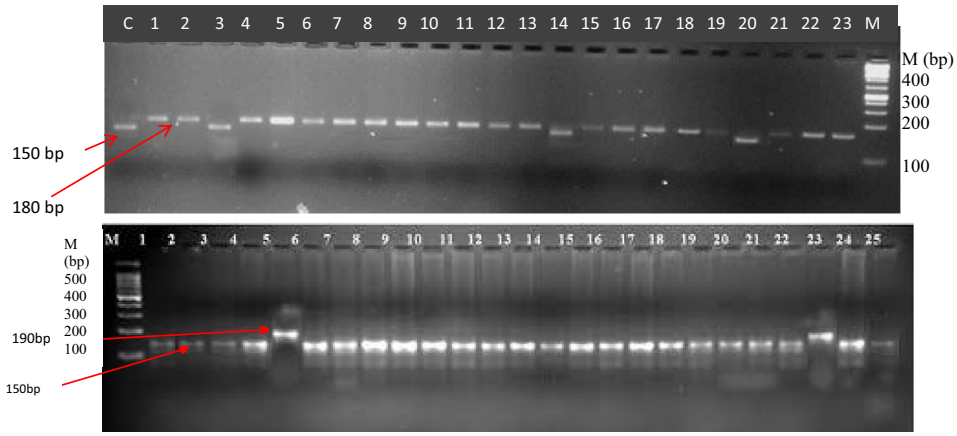
Analysis of variance revealed that significant difference among the four gynoecious genotypes for flowering and yield characters. The mean performance of the accessions for these characters is furnished (Table 4). The gynoecious line, IIHR-434, was early flowering at 29.6 days after planting at an earlier node (3.8 nodes) compared to other cucumber lines. The highest number of fruits was recorded in IIHR-435 (22 fruits) followed by IIHR-434 (19.7 fruits). The highest fruit length (19.2 cm), fruit girth (6.52 cm), fruit weight (224.9 gm) and yield per plant (3.6 kg) were recorded in IIHR-437 genotype. Our findings are in accordance with the results of Sharma et al. [30], Munshi et al. [24], Hanchinamani et al. [14] and Kumar et al. [18].

Validation of DNA Markers for Gynoecious Character in Cucumber

Twelve SSR primers were used to validate the gynoecious cucumber lines of F_4 population's, viz., IIHR-434, IIHR-435, IIHR-436 and IIHR-437 along with monoecious variety Swarna Agethi (Table 1). All twelve primers were amplified and separated on three percent agarose gel with ethidium bromide. Out of 12 SSR primers, two primers SSR-02021 and SSR-18718 differentiated the gynoecious plants from the monoecious plants and other SSR primers have shown monomorphic bands (Fig. 2). The SSR-02021

Table 4 Mean values for flowering and fruiting characters in gynoecious cucumber lines (F4)

Cucumber lines	Days to 1st female flower appearance	Node to 1st female flower appearance	Fruit length (cm)	Fruit girth (cm)	Fruit weight (gm)	Number of fruits/plant	Yield/plant (kg)
IIHR-434-3-8-5	29.6	3.8	14.8	5.70	172.5	19.7	3.39
IIHR-435-6-18-8	36.3	5.0	14.2	5.55	152.6	22.0	3.35
IIHR-436-2-7-13	31.9	3.2	16.3	5.47	161.3	16.7	2.69
IIHR-437-4-19-9	33.0	5.3	19.2	6.52	224.9	16.4	3.68
SE±	0.314	0.049	0.190	0.07	1.730	0.114	0.042
CD (5%)	1.020	0.158	0.617	2.40	5.612	0.371	0.138



(C- Monoecious line Swarna Agathi. M- 100bp ladder)

Fig. 2 Validation of SSR-18718 and SSR-02021 in F_4 population IIHR-437 and IIHR-434, respectively

marker has shown polymorphic bands for gynoecy and monoecious at 150 bp and 190 bp, whereas SSR-18718 at 180 bp and 150 bp, respectively, in all four F_4 populations. The genotyping data for individual plants were presented, and these are coexisted with phenotype of plants (Tables 5, 6). These two SSR markers will benefit MAS for gynoecious plants and will be useful for cucumber breeding program. Similar report of RAPD and SSR markers for

Table 5 Genotypic data for the F_4 population of the genotypes IIHR-434-3-8-5 and IIHR-435-6-18-8 (A–Gynoecious bands, B–Monoecious bands)

Plant numbers of IIHR-434-3-8-5	SSR-02021	SSR-18718	Plant numbers of IIHR-435-6-18-8	SSR-02021	SSR-18718
1.	A	A	1.	A	A
2.	A	A	2.	A	A
3.	A	A	3.	A	A
4.	A	A	4.	A	A
5.	B	B	5.	A	A
6.	A	A	6.	A	A
7.	A	A	7.	A	A
8.	A	A	8.	A	A
9.	A	A	9.	A	A
10.	A	A	10.	B	B
11.	A	A	11.	B	B
12.	A	A	12.	A	A
13.	A	A	13.	A	A
14.	A	A	14.	A	A
15.	A	A	15.	A	A
16.	A	A	16.	B	B
17.	A	A	17.	A	A
18.	A	A	18.	A	A
19.	A	A	19.	B	B
20.	A	A	20.	A	A
21.	A	A	21.	A	A
22.	A	A	22.	A	A
23.	B	B	23.	A	A
24.	A	A	24.	A	A
25.	A	A	25.	A	A
			26.	A	A

Table 6 Genotypic data for the F_4 population of the genotypes IIHR-436-2-7-13 and IIHR-437-4-19-9 (A–Gynoecious bands, B–Monoecious bands)

Plant numbers of IIHR-436-2-7-13	SSR-02021	SSR-18718	Plant numbers of IIHR-437-4-19-9	SSR-02021	SSR-18718
1.	A	A	1.	A	A
2.	A	A	2.	A	A
3.	A	A	3.	B	B
4.	A	A	4.	A	A
5.	A	A	5.	A	A
6.	A	A	6.	A	A
7.	A	A	7.	A	A
8.	A	A	8.	A	A
9.	A	A	9.	A	A
10.	A	A	10.	A	A
11.	B	B	11.	A	A
12.	A	A	12.	A	A
13.	A	A	13.	A	A
14.	B	A	14.	B	B
15.	A	A	15.	A	A
16.	A	A	16.	A	A
17.	A	A	17.	A	A
18.	A	A	18.	A	A
19.	A	A	19.	A	A
20.	A	B	20.	B	B
21.	A	A	21.	A	A
22.	A	A	22.	A	A
23.	B	B	23.	A	A
24.	A	A	24.	A	A
25.	A	A	25.	A	A
26.	A	A	26.	A	A
27.	A	A	27.	A	A
28.	A	A			
29.	A	A			
30.	A	A			
31.	A	A			
32.	A	A			

gynoecy identification in bitter gourd was published by Gaikwad et al. [11].

Conclusions

Homozygous gynoecious parthenocarpic cucumber lines from segregating populations could be isolated by consecutively selfing of individual plant selections. The gynoecious IIHR-434, IIHR-435, IIHR-436 and IIHR-437 were successfully developed under this study, where IIHR-437 has recorded higher yield among developed lines. GA_3 at 1.3 mM is an appropriate chemical for staminate flower induction in gynoecious cucumber. SSR-02021 and SSR-18718 markers associated with gynoecy are of great use to ascertain the purity of gynoecious lines at an early stage of development and also for a cost-effective breeding method.

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