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Characterization and maintenance of promising gynoecious bitter gourd line through hormonal regulation and micropropagation

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ABSTRACT

New gynoecious bitter gourd line, KAU-MCGy-101 identified from Kerala Agricultural University, India, was morphologically characterized. Silver thiosulphate (STS) was used for altering the sex expression of gynoecious line. STS induced hermaphrodite flowers in gynoecious plants and maximum number of hermaphrodite flowers was in plants receiving single spray at 200 ppm after emergence of first female flower. F_1 was generated by crossing with monoecious variety Preethi and all the hybrids were monoecious in nature. Expression of gynoecious character in the sib-mated gynoecious inbred and monoecy in F_1 hybrids, indicate the recessive nature of gynoecy in bitter gourd. Gynoecious inbred was also maintained through micropropagation. The shoot tip explants cultured on MS medium supplemented with 2.0 mgL^{-1} BA followed by 1.5 mgL^{-1} BA have given the highest shoot initiation response in 20.75 days. Multiple shoots, up to 5.50 per explant, were induced on MS medium with 2.0 mgL^{-1} BA and microshoots elongated best with 0.5 mgL^{-1} IAA + 0.5 mgL^{-1} NAA. Best *in vitro* rooting was on half strength MS medium with 3.0 gL^{-1} activated charcoal and 1.0 mgL^{-1} IBA. The gynoecious expression was found to be stable in tissue culture regenerated progenies.

Key words: *Momordica charantia* L., sex expression, micropropagation, genetics of gynoecy.

INTRODUCTION

Momordica charantia L. commonly known as bitter gourd, is a popular vegetable throughout tropics and subtropics of the world. Though monoecy is the predominant sex form in bitter gourd, gynoecy has been reported from India and China (Behera *et al*., 3). Similar to cucumber, gynoecy could be exploited in bitter gourd for developing hybrids with high sex ratio and for economizing F_1 hybrid seed production. Choice of gynoecious line as female parent will ensure high percentage of pistillate flowers in bitter gourd F_1s, with high yield potential and earliness (Rao *et al*., 20). However, no significant efforts are made so far to exploit heterosis with gynoecy. Since the availability and commercial utilisation of gynoecious lines are limited in bitter gourd, emphasis will be on development of agronomically superior gynoecious line and introgression of gynoecy into commercial inbreds. This study was taken up with the objective to characterize and investigate the genetics of gynoecy in the newly identified lines through sib mating and hybridization, to standardize the concentration and stages of application of silver thiosulphate for inducing maleness in the gynoecious lines and to establish the micropropagation protocol and to confirm the stability of sex expression in the field established progeny.

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MATERIALS AND METHODS

The study was carried out in the experimental field of Department of Vegetable Science, College of Agriculture, Kerala Agricultural University, during 2017-2020. Morphological characterization of the newly identified gynoecious line, KAU-MCGy-101 was done during the first season, based on minimal descriptors of agri-horticultural crops (NBPGR., 15). The monoecious variety 'Preethi' developed by KAU was used as male parent to develop cross with the gynoecious line.

Hormonal regulation of gynoecy using STS was studied during the second season. The effect of various concentrations of STS (150, 200, 250 ppm as single and double spray) on male flower induction was evaluated. First and second sprays of the double spray treatments were given at four-leaf stage and during the appearance of first pistillate flower, respectively. Single spray treatments were given after the appearance of first pistillate flower. Inbreds of gynoecious line were developed through sib mating of female flowers using pollen from the induced hermaphrodite flowers. Population generated from the sib mating between gynoecious plants and male sibs as well as the F_1 hybrid with 'Preethi' were evaluated during third season for the sex expression.

Micropropagation protocol was standardized to fix and maintain the gynoecious genotype. Vigorous,

pest and disease free gynoecious plants grown in the polyhouse have been selected as stock plants and tender shoot tip explants were collected in early morning hours. The explants were excised to 2.0-2.5 cm after removing immature leaves and leaf primordial. The explants were cultured on MS medium (Murashige and Skoog, 14) supplemented with various concentrations of BA and NAA for shoot initiation and multiple shoot induction (Table 1). For shoot elongation, MS medium with combinations of NAA and IAA was tried (Table 2). The elongated shoots (>5.0 cm) were excised and transferred to root induction media. Full and half strength MS media fortified with various concentrations of IBA and activated charcoal were used for *in vitro* rooting (Table 3). In all the treatments, pH of the medium was adjusted to 5.8± 0.1, solidified with 0.8% agar and autoclaved at 121°C at 15 psi (1.06 kg cm^{-2}) for 15-20 minutes. After explant inoculation, all the cultures were maintained at 27±1°C in an air conditioned culture room under 16/8 h photoperiod.

Table 1. Details of media composition for shoot induction.

Treatment	Media composition
T_0	MS + No hormone (Control)
T_1	MS + 0.5 mgl^{-1} BA + 0.1 mgl^{-1} NAA
T_2	MS + 0.5 mgl^{-1} BA + 0.2 mgl^{-1} NAA
T_3	MS + 1.0 mgl^{-1} BA + 0.1 mgl^{-1} NAA
T_4	MS + 1.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA
T_5	MS + 1.5 mgl^{-1} BA + 0.1 mgl^{-1} NAA
T_6	MS + 1.5 mgl^{-1} BA + 0.2 mgl^{-1} NAA
T_7	MS + 2.0 mgl^{-1} BA + 0.1 mgl^{-1}NAA
T_8	MS + 2.0 mgl^{-1} BA + 0.2 mgl^{-1}NAA
T_9	MS + 0.1 mgl^{-1}NAA
T_{10}	MS + 0.2 mgl^{-1}NAA
T_{11}	MS + 0.5 mgl^{-1} BA
T_{12}	MS + 1.0 mgl^{-1} BA
T_{13}	MS + 1.5 mgl^{-1} BA
T_{14}	MS + 2.0 mgl^{-1} BA

Table 2. Composition of shoot elongation media.

Treatment	Media composition
EM_1	MS + No hormone (Control)
EM_2	MS + 0.5 mgl^{-1} NAA
EM_3	MS + 1.0 mgl^{-1} NAA
EM_4	MS + 0.5 mgl^{-1} IAA
EM_5	MS + 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} NAA
EM_6	MS + 0.5 mgl^{-1} IAA + 1.0 mgl^{-1} NAA
EM_7	MS + 1.0 mgl^{-1} IAA
EM_8	MS + 1.0 mgl^{-1} IAA + 0.5 mgl^{-1} NAA
EM_9	MS + 1.0 mgl^{-1} IAA + 1.0 mgl^{-1} NAA

Table 3. Details of rooting media composition.

Treatment	Media composition
RM_1	½ MS + No hormone
RM_2	½ MS + 0.5 mgl^{-1} IBA
RM_3	½ MS + 1.0 mgl^{-1} IBA
RM_4	MS + No hormone
RM_5	MS + 0.5 mgl^{-1} IBA
RM_6	MS + 1.0 mgl^{-1}IBA
RM_7	½ MS + 1.0 mgl^{-1}IBA + 3.0 gl^{-1} Activated charcoal

The *in vitro* rooted plantlets were hardened in polythene bags containing cocopeat: soil: sand mixture (1:1:1) and kept in a mist chamber for 20 days. Established plants were transplanted to rain shelter for further growth and observations were recorded on sex expression.

RESULTS AND DISCUSSION

Gynoecious inbred was morphologically characterised using minimal descriptors and DUS test guidelines (Table 4). Fruits of the gynoecious inbred were dark green and spindle shaped with strong bitterness (Fig. 1). The shape of fruit at peduncle end was found to be acute in gynoecious inbred.

In bitter gourd, instead of male flowers STS application at various concentration induced hermaphrodite flowers. The induced hermaphrodite

Fig. 1. Gynoecious inbred KAU-MC-GY101 at fruiting stage.

Table 4. Qualitative and quantitative characters of sib mated gynoecious inbred KAU-MCGy-101.

Qualitative Characters		Quantitative characters	Range		Mean	CV (%)
			Min.	Max.		
Fruit skin colour	Dark green	Days to first female flower	21	40	32.78±1.66	18.96
Fruit skin lusture	Intermediate	Node at first female flower	15	27	20.07±0.96	17.96
Fruit: Shape of base at peduncle end	Acute	Days to first male flower	-	-	-	-
Fruit: Shape of apex at blossom end	Obtuse	Node at first male flower	-	-	-	-
Fruit: Shape in longitudinal section	Spindle shaped	Days to first harvest	55	73	65.25±1.56	8.94
Fruit surface	Deep tubercle	Number of harvests	3	5	3.64±0.17	17.38
Fruit: Tubercles	Many	Total number of fruits	12	25	15.57±0.95	22.91
Fruit: Tubercles prominence	Non-conspicuous	Total yield (kg)	0.87	2.08	1.25±0.09	27.20
Fruit: Ridge	Discontinuous	Average fruit weight (g)	60	102	76.36±2.91	14.24
Fruit bitterness	Strong	Average fruit length (cm)	12.04	15.38	13.55±0.28	7.83
		Average fruit girth (cm)	14.75	18.24	16.04±0.3	7.00

flowers were larger than the normal female flowers with prominent anthers (Figs. 2a, 2b, 2c), but not much variation was observed for the flower size between the treatments. Pollen grains when subjected to acetocarmine (1.0%) test, found completely stained with pinkish red colour, comparable to the pollen grain from normal male flower of Preethi (monoecious variety) which is an indication of male fertility, thus proving the efficacy of silver thiosulfate in inducing male fertility (Figs. 2d, 2e).

Among the six treatments of silver thiosulphate with different concentrations ranged from 150, 200 and 250 ppm, single spray of STS at 200 ppm after the first female flower emergence was found to be the best in terms of total number of hermaphrodite flowers produced (72.6). As per earlier reports, in bitter gourd STS application, when sprayed twice starting from 4 leaf stage at a concentration of

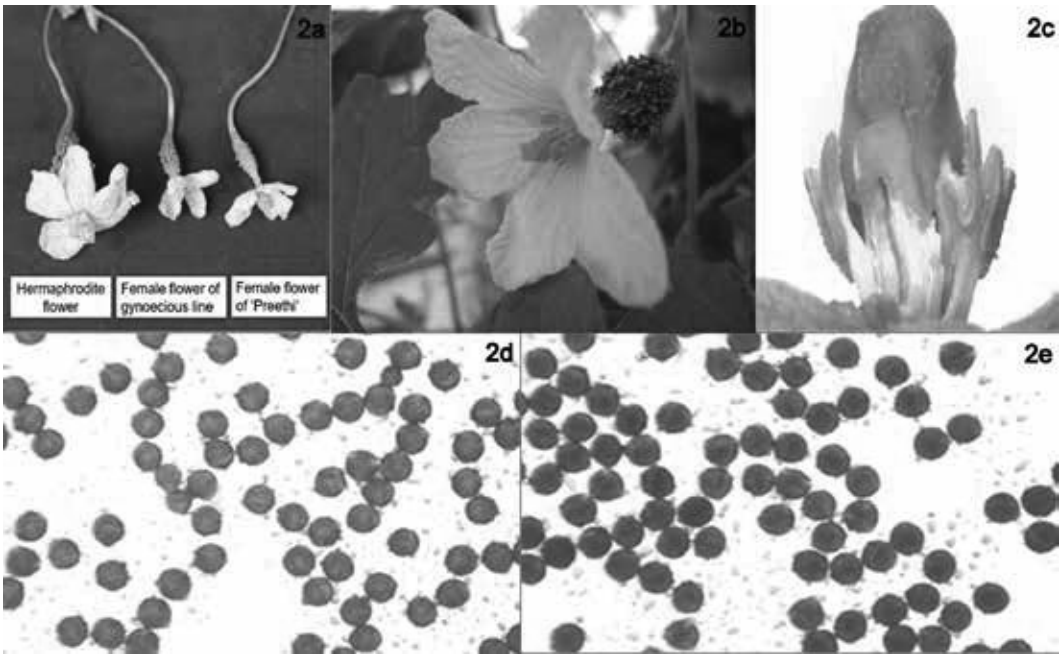

Fig. 2a -Comparison of female and hermaphrodite flower, 2**b-**Hermaphrodite flower, 2**c-**Anthers in hermaphrodite flower, 2**d-**Pollen from hermaphrodite flower, 2**e-**Pollen from male flowers of monoecious line.

6 mM resulted in highest percentage (57.63) of hermaphrodite flower, and it remained effective up to 15 days from application (Mishra *et al.,* 12). In the present study, all the treatments produced hermaphrodite flowers for a period of approximately three weeks (17.20-20.80 days) and then reverted back to original sex expression i.e., pistillate (Table 5). Hormonal treatments, may influence the flowering pattern of cucurbits, either by affecting the sexual differentiation of the floral bud or by a selective promotive or inhibitory effect on later development of the differentiated floral bud towards anthesis (Friedlander *et al*., 7). STS in general induces male flowers through the activity of silver ions. However, the present study revealed that in bitter gourd, instead of male flowers STS application induced hermaphrodite within two weeks of application. Mishra *et al.,* (11) obtained similar results, where in gynoecious bitter gourd, bisexual flowers appeared rather than male flowers after application of various concentrations of STS and GA_3. Ethylene, one of the growth regulators in plants, has been found to induce feminization. Most of the effects of ethylene can be antagonized by specific ethylene inhibitors such as GA_3, silver nitrate and silver thiosulphate. Silver ions (Ag^+) applied as silver thiosulphate are capable of generating ethylene insensitivity in plants by replacing copper ions (Cu^+) which are part of ethylene receptor and thus reducing ethylene sensitivity and enhancing staminate sex expression (Zhao *et al*., 24).

Normally sex differentiation in cucurbits occurred at two to four leaf stage (More and Sheshadri, 13) and growth hormones for altering the sex expression are recommended during two to four leaf stage. However present study revealed that application of STS at the later stage after the emergence of first female flower is more effective in inducing hermaphrodite flowers when compared to double application, one at two-four leaf stage and other at later stage. Thus breeder got the choice of altering the sex expression of the identified gynoecious line at the later stage of crop growth which holds huge potential in bitter gourd crop improvement. Earlier gynoecious lines reported from India produced hermaphrodite flowers only with the early application of growth regulator while KAU-MCGy-101 responded to late application of STS, which is a benison for breeding work.

The gynoecious plants produced female flowers throughout the growth period, indicating the stability of the gynoecious sex expression. In India, Ram *et al*. (17, 18) and Behera *et al*. (3) also reported stable gynoecious lines in bitter gourd. Gynoecious inbreds produced first female flower in the lower node. None of the inbreds produced male flowers while the hybrids were monoecious in nature. The inbreds generated by selfing through application of STS, produced fruits with a maximum weight of 102.0 g (Table 5). The inbred recorded an average fruit length and girth of 13.55 cm and 16.05 cm respectively. The range of biometric characters observed for the gynoecious inbred was also higher than the previous report (fruit weight 68.93 g, fruit length 8.87 cm) (Behera *et al*., 4). Thus, the gynoecious inbred, KAU-MCGy-101 holds enormous potential for future breeding programme for improving fruit character and yield in bitter gourd.

The F_1 hybrids developed by crossing the gynoecious line with monoecious variety Preethi had monoecious sex expression, with no segregation (Fig. 3) indicating the dominance of monecious trait over gynoecy. Manifestation of gynoecious trait in all inbreds generated through sib mating confirm the homozygous nature of gene controlling gynoecy. Uniformity in expression of gynoecious character in the sib mated gynoecious inbreds and monoecious character in F_1 hybrids indicate the homozygous recessive nature of the gynoecious trait. This is in

Table 5. Effect of different treatments of STS on gynoecious inbred for hermaphrodite flower induction.

Treatment	Days to first hermaphrodite flower anthesis	Node at which first hermaphrodite flower emerged	Branch on which first hermaphrodite flower emerged	Total number of hermaphrodite flowers	Duration of hermaphrodite flowering phase
T_1	13.0	22.4	2.4	38.6[b]	18.2
T_2	13.6	27.4	2.2	32.4[b]	18.2
T_3	13	26	2.2	68.4[a]	18.8
T_4	13.4	29.6	1.6	72.6[a]	20.8
T_5	13	28	2.2	47.8[b]	19.8
T_6	13.2	25.2	2.2	44.4[b]	17.2
CD 0.05	NS	NS	NS	19.12	NS
CV (%)	2.76	16.27	26.37	28.90	19.17

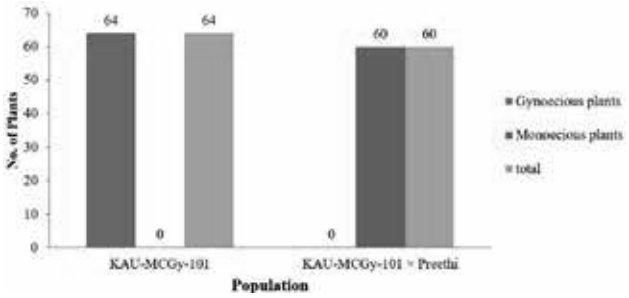

Fig. 3. Sex expression of sibmated gynoecious inbred and F_1 hybrid.

consonance with the previous reports that, gynoecy in bitter gourd is under the control of recessive gene (*gy-1*) (Ram *et al*., 19, Behera *et al*., 4). However analysis of F_2 data is required to confirm the number of genes and nature of gene action controlling gynoecy in the inbred KAU-MCGy-101.

Micropropagation was also attempted for maintenance of gynoecious inbred and maximum response was seen in cultures supplemented with BA alone (Figure 4a-b). Culture establishment and direct shoot proliferation of bitter gourd explants was found to be significantly influenced by the addition of BA in the medium. Out of the 15 treatments attempted, only six treatments showed shoot initiation response (Table 6). The treatment with 2.0 mgL^{-1} BA exhibited highest percentage (66.66) of shoot initiation within 21 days of inoculation and was significantly superior to all other treatments. *In vitro* growth regulation in plants and subsequent development are mainly controlled by the hormones auxins, cytokinins, and auxin-cytokinin interactions (Gaspar *et al*., 9). The shoot initiation response in this study is in accordance with the findings of Agarwal and Kamal (1) in bitter gourd. Highest percentage of shoot initiation (62) with an average of eight shoots per explant was noticed from shoot tip explants of cucumber when cultured on MS medium supplemented with BA alone (Vasudevan *et al*., 23).

Multiple shoot initiation was observed in shoot induction media (Figs. 4c, 4d). The treatment T_{14} (MS + 2.0 mgL^{-1} BA) produced maximum number of shoots per explant (5.50) and was on par with T_8 (MS + 2.0 mgL^{-1} BA + 0.2 mgL^{-1} NAA) and T_{12} (MS + 1.0 mgL^{-1} BA). Earlier reports suggested that a medium with combination of BA and NAA was the best to induce multiple shoots in bitter gourd (Al Munsur *et al*., 2). However, present study revealed that, addition

Table 6. Effect of BA and NAA on callusing and shoot initiation in bitter gourd (Mean±SE, n=12, NR – No Response, arc sin transformed value in parentheses).

Treatment	Days taken for callus initiation	Callus initiation response (%)	Days taken for shoot initiation	Shoot initiation response (%)	Shoots per explant
T_0	NR	0.00^{c} (1.43)	NR	NR	NR
T_1	4.25 ± 0.32^{d}	75.00^{ab} (64.52)	NR	NR	NR
T_2	4.17 ± 0.38^{d}	91.66^{ab} (79.05)	NR	NR	NR
T_3	4.08 ± 0.39^{d}	75.00^{ab} (60.00)	NR	NR	NR
T_4	3.33 ± 0.14^{d}	83.33^{ab} (69.52)	NR	NR	NR
T_5	4.00 ± 0.42^{d}	58.33^{b} (50.00)	NR	NR	NR
T_6	3.83 ± 0.29^{d}	83.33^{ab} (74.05)	20.50 ± 1.50^{b}	16.66^{cd} (8.9)	2.5 ± 0.50^{c}
T_7	3.58 ± 0.35^{d}	75.00^{ab} (60.00)	NR	NR	NR
T_8	3.67 ± 0.26^{d}	100.00^{a} (88.57)	19.33 ± 0.88^{b}	25.00^{bcd} (25.48)	4.3 ± 0.33^{ab}
T_9	NR	0.00^{c} (1.43)	NR	NR	NR
T_{10}	NR	0.00^{c} (1.43)	NR	NR	NR
T_{11}	11.16 ± 0.64^{a}	75.00^{ab} (60.00)	29.33 ± 2.03^{a}	25.00^{bcd} (25.48)	2.66 ± 0.33^{c}
T_{12}	9.58 ± 0.58^{b}	66.66^{ab} (59.52)	19.75 ± 2.01^{b}	33.33^{abc} (35)	4.25 ± 0.47^{ab}
T_{13}	8.33 ± 0.39^{c}	58.33^{b} (50.00)	28.71 ± 2.62^{a}	58.33^{ab} (50)	3.71 ± 0.42^{bc}
T_{14}	8.42 ± 0.51^{c}	58.33^{b} (50.00)	20.75 ± 1.73^{b}	66.66^{a} (59.52)	5.50 ± 0.62^{a}
CD (0.05)	1.15	32.75	5.26	26.63	1.3

T_0=MS+No hormone (Control); T_1=MS+0.5 mgl^{-1} BA+0.1 mgl^{-1} NAA; T_2=MS+0.5 mgl^{-1} BA+0.2 mgl^{-1} NAA; T_3=MS+1.0 mgl^{-1} BA+0.1 mgl^{-1} NAA; T_4=MS+1.0 mgl^{-1} BA+0.2 mgl^{-1} NAA; T_5=MS+1.5 mgl^{-1} BA+0.1 mgl^{-1} NAA; T_6=MS+1.5 mgl^{-1} BA+0.2 mgl^{-1} NAA; T_7=MS+2.0 mgl^{-1} BA+0.1 mgl^{-1} NAA; T_8=MS+2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA; T_9=MS+0.1 mgl^{-1} NAA; T_{10}=MS+0.2 mgl^{-1} NAA; T_{11}=MS+0.5 mgl^{-1} BA; T_{12}=MS+1.0 mgl^{-1} BA; T_{13}=MS+1.5 mgl^{-1} BA; T_{14}=MS+2.0 mgl^{-1} BA

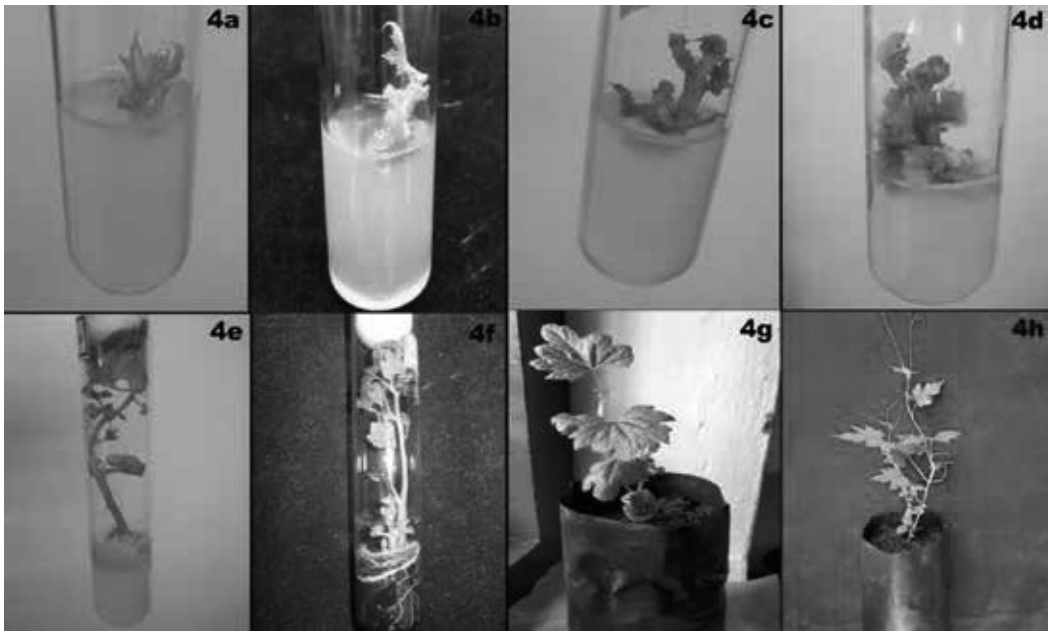

Fig. 4a- Direct organogenesis in MS media supplemented with 2.0 mgL^{-1} BA, **4b-**Direct organogenesis in MS media with 1.5 mgL^{-1} BA, **4c** and **4d-**Multiple shoot initiation in MS media with 2.0 mgL^{-1} BA, **4e-**Shoot elongation in MS media with 0.5 mgL^{-1} IAA+0.5 mgL^{-1} NAA, **4f-** In vitro rooting, **4g** and **4h-**Planting out and hardening of tissue culture plants.

of BA alone favoured multiple shoot induction from the shoot tip explants of bitter gourd. Adventitious bud proliferation was promoted by cytokinin in the *in vitro* cultures of bitter gourd (Song & Gao, 22). The efficiency of cytokinin in inducing shoot regeneration and multiple shoot induction in *Momordica dioica* (Roxb.) has also been reported by Devendra *et al*., (5).

Among the various treatments tried for *in vitro* shoot elongation, MS medium supplemented with 0.5 mgL^{-1} IAA and 0.5 mgL^{-1} NAA was significantly superior to all other treatments (Fig. 4e). Maximum shoot length (9.03 cm) for microshoots after one month was obtained from this treatment combination (Fig. 5a). Unlike earlier reports where, various combinations of IAA and BA used to achieve shoot elongation (Al Munsur *et al*., 2), MS medium supplemented with 0.5 mgL^{-1} IAA and 0.5 mgL^{-1} NAA was the best treatment for *in vitro* shoot elongation. Auxin favoured elongation under *in vitro* condition, and the role of auxin is manifested in the present study for promoting *in vitro* shoot elongation.

Out of the seven treatments compared, *in vitro* rooting was obtained from only two treatments viz., RM_7 and RM_3 (Fig. 5b). Addition of activated charcoal in the rooting medium along with IBA was found to enhance root initiation in the *in vitro* shoots of bitter gourd (Fig. 4f). *In vitro* regeneration of adventitious roots was the best in half strength MS medium augmented with 1.0 mgL^{-1} IBA and 3.0 gL^{-1} activated charcoal (RM_7). Maximum rooting response (100%) and early root initiation (12 days) was observed with this treatment combination (Fig. 5c). IBA and charcoal combination was found to be the best for inducing maximum number of roots (7.0). Efficiency of IBA in root induction of bitter gourd has already been reported (Al Munsur *et al*., 2). Addition of activated charcoal in the rooting medium along with IBA was found to enhance root initiation in the *in vitro* shoots of bitter gourd. These results corroborate the findings of Saha and Behera (21) in bitter gourd, as the addition of activated charcoal in the rooting medium improved overall rooting capacity of mature explants. Pradeepkumar *et al*. (16) obtained a similar result from *in vitro* culturing of male sterile ridge gourd plants, as highest percentage of rooting (95%) was observed on half strength MS medium supplemented with 1.0 mgL^{-1} IBA and 200 mgL^{-1} charcoal. Supplementation of rooting medium with activated charcoal also proved to be excellent in terms of earliness in root induction, root number, and length as well as response to rooting of *in vitro* generated microshoots (Gantait *et al.,* 8). The promotion of rooting by activated charcoal can be interpreted considering two facts; the adsorption of inhibitory substances in the culture medium (Fridborg *et al*., 6) and establishment of a darkened environment in medium which is conducive to the accumulation of photosensitive auxin or cofactors. Full strength MS medium was mostly used for rooting in bitter melon,

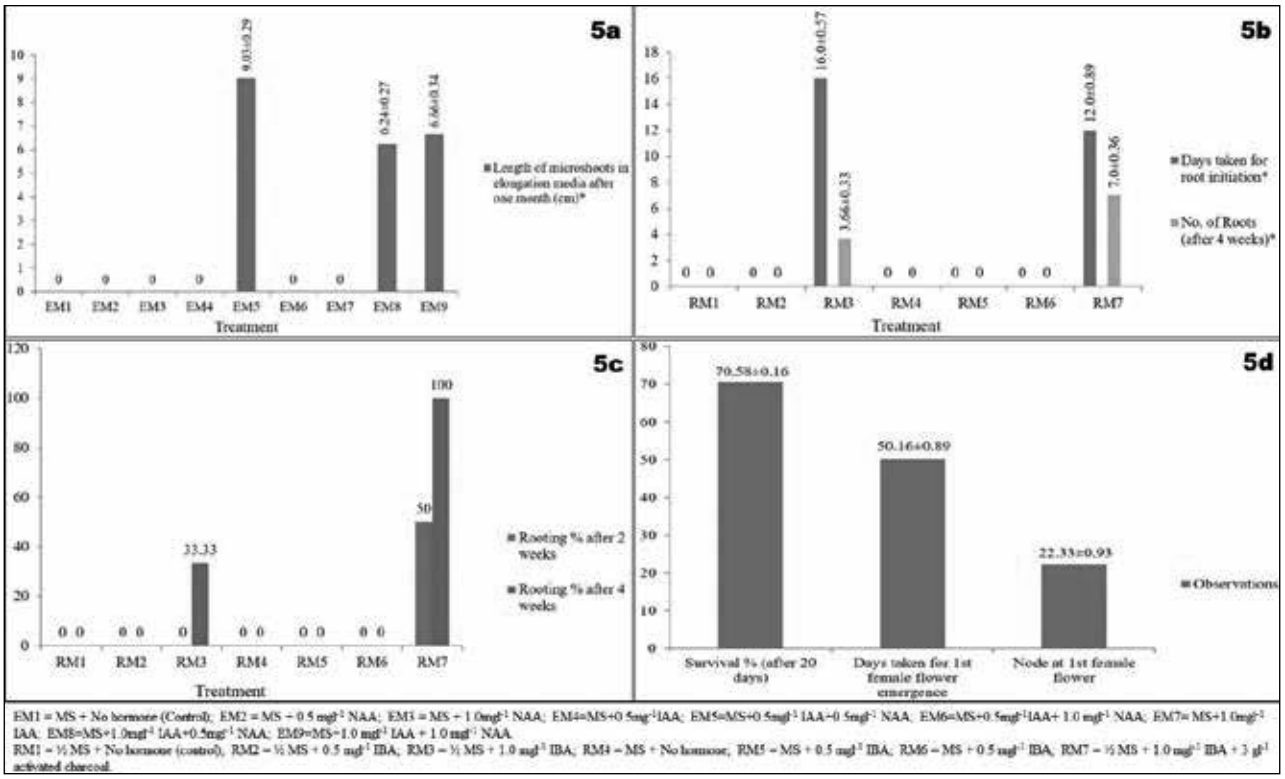

Fig. 5a- Effect of NAA and IAA on *in vitro* shoot elongation, **5b-**Effect of composition of media with IBA and activated charcoal on *in vitro* rooting, **5c-**Effect of composition of media with IBA and activated charcoal on rooting frequency, **5d-**Gynoecious sex expression of hardened plantlets.

but Liu *et al*. (10) observed excellent rooting in medium with half concentration of macronutrients.

All the *in vitro* rooted plantlets were hardened in sterilized potting medium and kept inside the mist chamber for 20 days (Figs. 4g, 4f). Survival per cent was found to be 70.58 (Fig. 5d). Unlike earlier studies where the micropropagation protocol was standardized upto the initial survival percentage in the field, present investigation evaluated the gynoecious sex expression of the *in vitro* regenerated plants. All the tissue culture regenerated plants produced only female flowers and the ex vitro hardened plants produced flowers a bit late (50.16 days after planting out) compared to seed propagated gynoecious plants (32.78 days). However there was not much difference in number of nodes taken for female flower emergence which was 22.33 for tissue culture plants and 20.07 nodes for seed propagated plants. It was found that earlier exposure of the explant to high dose of cytokinin or auxin had in no way influenced the sex expression of the gynoecious line, and thus proved the efficiency of developed protocol and stability of the gynoecious sex expression.

The new gynoecious line KAU-MC-GY101 is superior to the other reported gynoecious line from India and amenable to *in-situ* and *in-vitro* maintenance. The gynoecious expression is stable and hermaphrodite flower could be induced through STS spray even at the late stage unlike other gynoecious genotypes so far reported. The gynoecious expression is stable in tissue culture regenerated progenies and the initial studies revealed recessive nature of gynoecy operating in the gynoecious line.

AUTHORS' CONTRIBUTION

Conceptualization of research (PT, MAJ, DM); Designing of the experiments (PT, MAJ); Contribution of experimental materials (VR, VK); Execution of field/lab experiments and data collection (RPK, VK); Analysis of data and interpretation (PT, MAJ); Preparation of the manuscript (MAJ, PT).

DECLARATION

The authors declare that they have no conflict of interest.

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