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Induction of Fertile Male Flowers in Genetically Female *Cannabis sativa* Plants by Silver Nitrate and Silver Thiosulphate Anionic Complex

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Summary. Apical application of silver nitrate ($AgNO_3$; 50 and 100 µg per plant) and silver thiosulphate anionic complex ($Ag(S_2O_3)_2^{3-}$; STS; 25, 50 and 100 µg per plant) to female plants of *Cannabis sativa* induced the formation of reduced male, intersexual and fully altered male flowers on the newly formed primary lateral branches (PLBs); 10 µg per plant of $AgNO_3$ was ineffective and 150 µg treatment proved inhibitory. A maximum number of fully altered male flowers were formed in response to 100 µg STS. The induced male flowers produced pollen grains that germinated on stigmas and effected seed set. Silver ion applied as STS was more effective than $AgNO_3$ in inducing flowers of altered sex. The induction of male flowers on female plants demonstrated in this work is useful for producing seeds that give rise to only female plants. This technique is also useful for maintaining gynoecious lines.

Key words: *Cannabis sativa* – Sex expression – Silver nitrate – Silver thiosulphate anionic complex

Introduction

Sex expression in flowering plants is regulated by genetic, environmental and hormonal factors. There is evidence that specific endogenous hormones play an important role in maintaining the genetic sex, and that sex can be modified through exogenous growth regulators, especially in the sexually polymorphic systems (Heslop-Harrison 1964). In general gibberellins favour male sex expression and auxin, ethylene and cytokinins promote female sex expression in various monoecious and dioecious systems (Mohan Ram 1980). Treatments which reduce the ethylene level in the tissues (hypobaric conditions, treatment with benzothiodiazole) or antagonize the action of ethylene (CO_2) cause the formation of male or bisexual flowers in place of female ones (Byers et al. 1972).

Recently silver ion has been shown to interfere with ethylene action, presumably at the ethylene receptor sites (Beyer 1976a). Following the report by Beyer (1976b) that the application of silver nitrate ($AgNO_3$) initiates male flower formation in gynoecious cucumber, it has been recently shown in four cucumber lines that silver ion is superior to GA_3 for male flower induction (Kalloo 1978; Tolla and Peterson 1979). In the pistillate 240 line of *Ricinus communis* (Ankineedu and Rao 1973) an internodal injection of aqueous silver nitrate solution induced fertile male flowers on the strictly pistillate primary terminal raceme (Mohan Ram and Sett 1980). Using labelled silver (110^mAg), Veen and van de Geijn (1978) showed that silver applied as silver thiosulphate anionic complex (STS) is transported faster (2^{mh-1}) than $AgNO_3$ (3 cm day^{-1}) and that it completely counteracts the ethylene effect and significantly extends the vase-life of carnations (Veen 1979).

A preliminary report from this laboratory showed that apical application of silver nitrate (100 µg/plant) induces fertile male flowers in female *Cannabis* plants (Sarath and Mohan Ram 1979). The present investigation was undertaken with the objective of establishing the minimal and optimal dosage of $AgNO_3$ required to modify the sex expression of female plants of *Cannabis sativa* and also to find out whether STS acts as an ethylene antagonist in male sex induction.

Material and Methods

Seedlings of *Cannabis sativa* growing naturally in the Botanical Garden of the Department were transplanted at the 3- or 4-leaf stage to 25 cm wide earthenware pots filled with garden soil, in November, 1980. The sex of the plants was determined after flower initiation. Only female plants were selected for study. These plants have a pair of sessile female flowers at the

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base of each leaf. Each female flower bears a boat-shaped, green, glandular perigynous bract. Inside the bract lies the ovary, whose base is partly surrounded by a papery cupule. The style is highly reduced and bears two elongated unequal papillate stigmas (Fig. 1a inset). In contrast, the male flowers (on male plants) are borne in clusters (15–17 flowers at each node) and bear five tepals and five stamens.

Either an aqueous solution of $AgNO_3$ or of silver thiosulphate anionic complex $[Ag(S_2O_3)_2^{3-}$; (STS); 1 silver nitrate ($AgNO_3$); 8 sodium thiosulphate ($Na_2S_2O_3$) w/w] was applied to the growing shoot tip of the female plants with the help of a 0.01 ml pipette. Tween-80 (0.01%) was used as the surfactant. A 10 µl drop of the test compound was applied each day for 5 days to make up the total amount. Ten plants were maintained for each treatment. The plants had received 50, 100, 150 µg/plant of $AgNO_3$ or 25, 50, 100 µg/plant of STS each, by the end of the fifth day. The control plants received the surfactant solution only. The viability of the pollen grains was tested either by immersing the pollen in 2,3,5-triphenyl 2 H-tetrazolium chloride (TTC; Nutritional Biochemical Corporation, Cleveland, Ohio, USA) for 3 to 5 min or by germinating the freshly collected pollen from the induced male flowers in a medium consisting of 7% sucrose and 1% agar.

Results

In response to the 50 and 100 µg treatments, the young leaves covering the shoot apex turned black (after the final treatment), giving a burnt appearance. Apical growth was suspended and the shoot tip resumed its activity 20–25 days after the final treatment. The increment in height as ascertained 48 days after treatment, showed no marked difference from that of the controls (Table 1). The number of nodes was significantly higher in the treated plants (Table 1). On account of a temporary cessation of growth of the shoot meristem in the above two treatments, apical dominance was released and the primary lateral branches (PLBs) elongated, surpassing the length of those present in the control plants (Fig. 1a). These branches bore flowers of the following sex types: (i) female (♀); (ii) intersexual (⚥; flowers bearing both female and male organs Fig. 1d); (iii) reduced male (R♂; flowers having four or fewer stamens, Fig. 1e) and (iv) male (♂; bearing 5 stamens with a copious amount of pollen grains, Fig. 1f). Figure 1b shows an excised PLB from the 100 µg treatment, in which induced male flowers are seen. A minimum of eight newly formed PLBs bearing flowers of altered sex were present in all treated plants. Data collected from these have been presented in Table 1. There was a distinct change in the sex of the flowers appearing on the main axis after treatment but in view of the larger number of flowers formed on the PLBs, data were collected only from these. The total number of flowers formed on each PLB was higher in the treated plants than in the controls. This was because of the greater lengths of the PLBs on the treated plants and also because of higher flower number per node (as compared to the restricted number of female flowers at each node on the control plants). Surprisingly, with both the treatments an increase in the percentage of flowers bearing an altered sex (out of the total number of flowers) was noticed from position 1 to 8 of the PLBs (from base upwards), (Table 1).

The shoot tip became black, dried up completely and failed to revive in response to the highest amount of $AgNO_3$ applied (150 µg per plant). The little increment in height that occurred was due to internodal elongation (Table 1). The young leaves already present became yellow and abscised without further expansion. The suppressed primary lateral branches arising at the lower nodes of the main axis became highly stimulated and caused the plants to become bushy. Surprisingly, these branches bore only a few abortive female flowers.

In another experiment it was found that 10 µg $AgNO_3$ was ineffective in modifying sex expression and 25 µg treatment caused only the first three newly formed PLBs (from base upwards) to bear a maximum number of flowers of altered sex along with normal female flowers. However, the percentage of flowers bearing altered sex along each PLB in plants treated with 25 µg was lower than that formed in 50 µg treatment.

Three amounts of STS, namely 25, 50 and 100 µg per plant, were selected on the basis of previous experience with $AgNO_3$. The shoot tips of the treated plants became black and appeared dry in response to all the three dosages of STS. The young leaves present at the time of treatment became decolourised and their shapes changed drastically at maturity. The average leaf area in control, 25, 50 and 100 µg treatments were 30.12, 14.46, 9.64 and 6.02 cm^2 respectively. The shoot tip resumed its growth 20–25 days after treatment with 25 and 50 µg STS, whereas when treated with 100 µg, it failed to recover. The newly formed leaves in the former two treatments were also small and deformed. Treatment with 25 µg of STS caused only a marginal stimulation in height, although the node number was nearly doubled (Table 2). In plants that were given 50 µg of the compound, the height was significantly lower than that of the control, although the node number remained unchanged. In response to 100 µg of STS, the shoot tip ceased to grow further and the small increment in height resulted entirely from internodal elongation (Table 2). It may be inferred that cell elongation was more drastically affected than cell division in response to 25 and 50 µg per plant. In these treatments the upper nodes produced PLBs with flowers of altered sex (Table 2). Data presented in Table 2 pertain to the first eight PLBs formed after treatment, since a maximum number of flowers bearing altered sex were present on them. Although the length

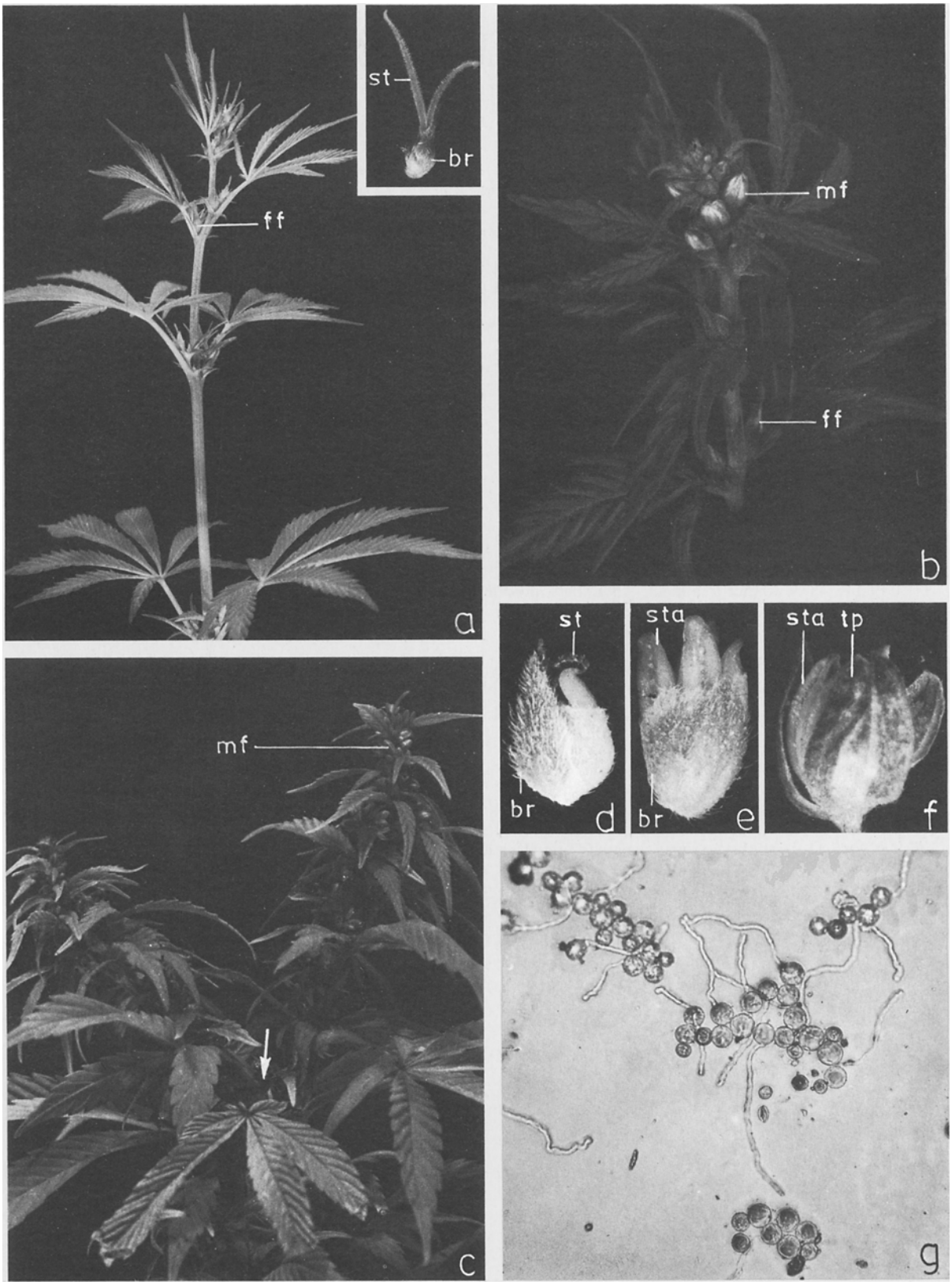

Fig. 1a–g. *Cannabis sativa:* **a** Terminal portion of a control female plant ×0.6; inset shows an enlarged female flower, note the prominent, unequal stigmas (st) projecting out of the bract (br), ×2; **b** Excised PLB from a plant treated with 100 μg $AgNO_3$. Note the female flowers (ff) at the lower nodes and male flowers (mf) at the upper nodes, ×2; **c** Apical portion of a 100 μg STS-treated plant. Arrow indicates the dried shoot tip. Male flowers (mf) are seen in clusters on the PLBs, ×1; **d** An intersexual flower showing an anther bearing a stigma (st) and the hairy bract (br) enclosing the female flower, ×5; **e** Reduced male flower with three stamens (sta) projecting beyond the bract, typical of a female flower, ×5; **f** A fully altered male flower showing five tepals (tp) and five stamens (sta). The bract has been eliminated, ×7; **g** Germinating pollen grains obtained from male flowers induced by $AgNO_3$ (100 μg) treatment (2 h after incubating in agar-sucrose medium), ×166

Table 1. Effect of $AgNO_3$ on shoot growth, length of primary lateral branches and flower sex expression[a]

	Treatments (µg/plant)									
	0 (control)					50				
	$\bar{x}$		CI			$\bar{x}$				CI
Increment in height (cm)	34.9		7.59			33.95				6.45[NS]
Increment in the number of nodes	7.0		0.45			11.11				2.14**
Position of primary lateral branches[b]	Length (cm)		Flower number	Length (cm)		Flower number				Percentage of altered flowers[c]
	$\bar{x}$	CI	(♀)	$\bar{x}$	CI	♀	♀♂	R♂	♂	
1	3.90	0.82	9.20	4.55	0.80	5.8	3.5	1.0	5.0	62.09
2	4.14	0.78	7.90	3.50	0.77	6.6	1.0	2.0	7.0	62.50
3	2.68	0.59	10.60	4.40	0.71	5.3	1.2	1.5	4.0	55.83
4	2.27	0.30	7.40	3.10	0.71	4.8	2.0	1.5	3.6	59.66
5	2.31	0.37	6.60	1.90	0.53	3.4	1.5	2.5	3.0	67.31
6	3.07	1.10	5.30	[d]	[d]	3.0	1.6	1.0	2.5	62.96
7	3.20	2.04	6.50	[d]	[d]	3.3	1.5	1.5	3.0	64.52
8	2.30	1.50	7.30	[d]	[d]	2.0	1.0	4.0	4.0	81.82

[a] Average of 10 plants, 48 days after treatment; [b] Numbers indicate position from base upwards; [c] Percentage of flowers bearing altered sex out of the total number of flowers; [d] Length < 1 cm; [e] Dried shoot tip;
** Highly significant over control at $P \leq 0.01$; NS = Not significant; CI = Confidence interval at $P \leq 0.05$

Table 2. Effect of $Ag(S_2O_3)_2^{3-}$ on shoot growth, length of primary lateral branches and flower sex expression[a]

	Treatments (µg/plant)									
	0 (control)					25				
	$\bar{x}$		CI			$\bar{x}$				CI
Increment in height (cm)	34.9		7.59			36.1				7.19[NS]
Increment in the number of nodes	7.0		0.45			13.0				1.84**
Position of primary lateral branches[b]	Length (cm)		Flower number	Length (cm)		Flower number				Percentage of altered flowers[c]
	$\bar{x}$	CI	(♀)	$\bar{x}$	CI	♀	♀♂	R♂	♂	
1	3.90	0.87	9.2	4.50	1.76	5.8	1.0	2.5	7.2	64.85
2	4.14	0.87	7.9	5.20	1.68	6.2	1.0	1.0	3.7	47.90
3	2.68	0.59	10.6	2.60	0.69	6.5	1.0	2.0	6.8	60.12
4	2.27	0.30	7.4	3.45	1.33	4.3	1.0	2.0	5.0	65.04
5	2.37	0.37	6.6	2.00	0.69	5.9	1.5	3.5	7.8	68.45
6	3.07	1.10	5.3	[d]	[d]	4.7	1.0	2.0	6.5	66.90
7	3.20	2.04	6.5	[d]	[d]	2.7	1.0	2.0	4.0	72.16
8	2.30	1.50	7.3	[d]	[d]	5.7	1.5	1.0	6.2	60.42

Footnotes see Table 1

Table 1. (continued)

	100					150	
	x̄				CI	x̄	CI
	32.69				10.45[NS]	2.25	1.04**
	9.66				1.47**	[e]	

Length (cm)		Flower number				Percentage of altered flowers[c]	Floriferous branches absent
x̄	CI	♀	⚥	R♂	♂		
8.00	1.01	3.9	3.0	5.0	7.0	51.90	
7.10	4.44	2.1	2.0	2.5	8.0	50.80	
6.90	2.12	9.5	3.6	4.5	7.2	61.69	
4.90	2.69	8.5	1.0	2.0	4.6	47.20	
340	1.66	7.2	2.8	3.2	6.1	62.69	
[d]	[d]	4.4	1.8	3.0	7.6	73.81	
[d]	[d]	3.9	1.8	3.5	6.8	75.62	
[d]	[d]	1.8	1.6	2.5	6.3	85.25	

Table 2. (continued)

	50					100	
	x̄				CI	x̄	CI
	18.72				5.07**	4.29	1.58**
	7.20				2.84[NS]	[e]	

Length (cm)		Flower number				Percentage of altered flowers[c]	Length (cm)		Flower number				Percentage of altered flowers[c]
x̄	CI	♀	⚥	R♂	♂		x̄	CI	♀	⚥	R♂	♂	
5.88	2.42	4.8	2.0	1.0	6.0	65.22	25.60	12.47	17.5	9.4	12.0	68.2	83.66
3.13	1.29	3.2	1.0	4.3	6.8	79.08	16.20	12.47	7.7	3.0	2.3	22.5	78.31
2.88	2.09	2.4	2.0	2.0	7.3	82.48	14.58	6.18	9.2	2.0	2.3	20.2	72.70
3.25	1.93	2.2	1.3	2.0	3.3	75.00							
1.63	0.62	1.7	2.0	1.0	5.2	82.83							
[d]	[d]	2.0	1.0	2.0	5.6	81.83							
[d]	[d]	2.0	1.0	2.0	6.0	81.82							
[d]	[d]	2.0	2.0	1.0	6.0	81.82							

of these branches was not significantly different from that in the controls, the total number of flowers per branch in the treated plants was higher, resulting in an aggregation of flowers at the nodes. In the treatment with 50 µg, the number of female flowers formed on each PLB was less and the percentage of altered flowers was more than that formed in response to 25 µg. In plants receiving 100 µg, the first three nodes (which had no PLBs at the time of treatment) put out highly elongated PLBs which bore a large number of flowers of altered sex along with some female flowers (Table 2). Interestingly, the number of fully altered male flowers was significantly greater than that of the reduced males, intersexual and female flowers. Also, in each branch the number of altered flowers was higher in the upper nodes, resulting in the clustering of male flowers towards the apex. The percentage of altered flowers out of the total number of flowers present on each branch was much higher in the 100 µg (Fig. 1 c) treatment than that in the 25 and 50 µg treatments.

Irrespective of the extent of masculinization (whether male, reduced male or intersexual condition) caused by treatment with either $AgNO_3$ or STS, the anthers in the flowers of altered sex contained a large quantity of viable pollen grains as ascertained by the tetrazolium test. The viable pollen grains also germinated within half an hour of incubation in agar-sucrose medium (Fig. 1 g). To test the ability of pollen for germination and for effecting seed set, the stigmas of unpollinated female flowers were pollinated with the pollen from induced male flowers and bagged. The handpollinated female flowers developed seeded fruits, whereas those left unpollinated and bagged failed to do so and abscised. Thus pollen from induced male flowers were capable of inducing seed set. On the basis of our previous experiments (Jaiswal 1972) we presume that the progeny of these seeds would be 100% pistillate, although in this particular experiment the sex of the plants has not been scored.

Discussion

The present investigation has substantiated the earlier work done in this laboratory by Sarath and Mohan Ram (1979) and has established that apical application of $AgNO_3$ to the female plants of *Cannabis* stimulates the development of male flowers. Additionally, the experiments have shown that 50 and 100 µg amounts of $AgNO_3$ are most effective in inducing fertile male flowers at the newly formed nodes on the main axis and also on the freshly formed PLBs. Treatment with 10 µg proved ineffective whereas application of 150 µg strongly inhibited the growth of the apical and lateral meristems.

Silver ion applied as $AgNO_3$ was shown by Beyer (1976 a) to block the action of the exogenously applied ethylene. He demonstrated this phenomenon in the classical 'triple' response (which included growth retardation, stem swelling and horizontal growth) in intact etiolated peas; in leaf, flower and fruit abscission in cotton, and in the senescence of *Cattleya*. In a gynoecious cucumber plant, $AgNO_3$ effectively shifted the sex expression from female to male (Beyer 1976 b). Intersexual and staminate flowers were induced by $AgNO_3$ treatment in other gynoecious cucumber lines by Kalloo (1978), Atsmon and Tabbak (1979) and Tolla and Peterson (1979). Curiously in a monoecious cucumber, $AgNO_3$ nullified the effect of mechanical stress and induced pistillate flower production (Takahashi and Suge 1980).

The present work has also shown that STS is more effective than $AgNO_3$ in inducing male flowers on female plants. The number of male flowers induced per plant and the percentage of flowers of altered sex along each PLB were higher in STS treatment. STS counteracted the ethylene effect in carnations and extended their vase-life significantly (Veen 1979). In the same plant, a 10-minute pulse treatment with STS (0.1 mM Ag) doubled the vase-life of the flowers (Reid et al. 1980). Dimalla and Van Staden (1980) have also demonstrated that the shelf-life of carnations can be dramatically increased by immersing the cut end in STS for 10 min. The present study has indicated that application of silver in the anionic complex is more effective than that in the cationic form. Additionally the present investigation has clearly demonstrated that STS also triggers male sex expression in female plants of *Cannabis sativa* probably by blocking the action of ethylene. Chemical induction of male flowers is thus a means of producing guaranteed female plants or of maintaining gynoecious lines through the production of seeds following selfing in female plants. If properly exploited, this technique should be highly rewarding in crop improvement programmes.

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