Korean J. Plant Res. 33(6):682-688(2020)
https://doi.org/10.7732/kjpr.2020.33.6.682

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Effect of Timing of Ethephon Treatment on the Formation of Female Flowers and Seeds from Male Plant of Hemp (*Cannabis sativa* L.)

Youn-Ho Moon[1], Yoon Jeong Lee[1], Sung Cheol Koo[2], Mok Hur[2], Yun Chan Huh[3], Jae-Ki Chang[3] and Woo Tae Park[2]*

[1]Post-doc, [2]Researcher and [3]Senior Researcher, Department of Herbal Crop Research, NIHHS, RDA, Eumseong 27709, Korea

Abstract - Hemp (*Cannabis sativa* L.) is a dioecious plant, although monoecious plants are bred in some cultivars for fiber or seed production. Recently, hemp has received attention for medicinal use with some cannabinoids, including cannabidiol. Self-fertilization for breeding inbred lines is difficult because of dioeciousness and anemophily in hemp. This experiment was conducted to develop a self-fertilization method by forming female flowers and seeds from male plants of dioecious hemp. To induce the formation of female flowers on male plants, 500 ㎎ L^{-1} of ethephon was sprayed on plants at soon, seven and fourteen days after primordia formation. The plant ratio of female flowers formation and the number of harvested seeds were increased by ethephon treatment. Female flowers of male plants have 5 stigmas in contrast to the dual stigma of female 1plants. Male plant seeds were lighter and smaller than those from female plants. Although the germination rate was lower than that of normal seeds from female plants, the seeds from male plants germinated to grow seedlings. Thus, we suggest that hemp plants should be treated with ethephon at soon after primordia formation to induce the formation of more female flowers on the male plants.

Key words – Breeding, Cannabinoids, Female flowers, Male plants, Primordia

Introduction

Hemp (*Cannabis sativa* L.) originated from Central Asia and has been cultivated for fiber production for thousands of years in Korea (Moon *et al*., 2006). Hemp seeds have been used for the production of functional food and cosmetics in Europe and North America because of its rich non-saturated fatty acids, which include γ-linolenic acid (Moon *et al*., 2005). Hemp belongs to the family Cannabaceae and an anemophilous plant that is pollinated by wind (Cabezudo *et al*., 1997; McPartland, 2018; Small, 2015).

There are some debates about the speciation of hemp regarding whether they are divided into two species (*C. sativa* and *C. indica*) or one species (Clarke and Merlin, 2016). However, most researchers believe that *C. indica* and *C. sativa* are one species because there are no barriers for hybridization between the species by pollination (Beutler and Marderosian, 1978; de Meijer and Van Soest, 1992; McPartland, 2018). Recently, hemp has received attention for medicinal use with cannabinoids produced in fertilized female flowers (Chandra *et al*., 2017). The cannabinoids are synthesized at glandular trichomes which are abundant on bracts of female flowers (Clarke and Merlin, 2016; Kim, 2019). The cannabinoid content of bracts were highest in maturing stage but decreased with being senescent (Mahlberg and Kim, 2004; Zager *et al*., 2019). The seed borne bracts were early senescent with maturing seeds whereas non-seeded bracts were late senescent (Clarke and Merlin, 2016). Thus, for the production of medicinal cannabinoids, female plants of dioecious cultivars should be cultivated exclusively to prevent fertilization with pollen from male flowers of male or monoecious plants (Chandra *et al*., 2017; Spitzer-Rimon *et al*., 2019).

Separate sexes were evolved from hermaphroditic ancester having both stamens and carpels in the same flower in plantae (Aryal and Ming, 2014; Bai *et al*., 2019; Moliterni *et al*., 2004). Although there are some monoecious cultivars that produce

*Corresponding author. E-mail : harusarinamu@korea.kr
Tel. +82-43-871-5686

male and female flowers on the same plant, most of hemp cultivars are dioecious that produces male (pollen) flowers in male plants and female (seed) flowers in females (Clarke and Merlin, 2016; de Meijer and Van Soest, 1992; Mandolino *et al*., 1999). The gender of dioecious plants is regulated by genetic, epigenetic and hormonal factor (Bai *et al*., 2019; Heikrujam *et al*., 2015). Hemp have nine pair of autosomes and one pair of sexual chromosomes which are X and Y (Moliterni *et al*., 2004). Male plants of hemp are heterogametic (XY) whereas females are homogametic in sexual chromosome pairs (Heikrujam *et al*, 2015; Moliterni *et al*., 2004). The sex expression of dioecious plants can be changed by the plant hormones, and ethylene is a feminizing hormone in the plantae (Heikrujam *et al*, 2015; Sakthinathan *et al*, 2017). Thus, hormone application can induce partial sexual change in dioecious plants (Aryal and Ming, 2014; Heikrujam *et al*., 2015).

Some breeders bred medicinal cultivars that have a large content of cannabidiol, the major medicinal cannabinoid. However, most of the cultivars were not inbred lines, but F_1 or selected lines from unknown parents and therefore the cultivars should be propagated not by seeds but by vegetative propagation (Carter, 2017; Cohen, 2014, 2020; Holmes, 2019; Lewis, 2020). The effective methods to breed inbred lines are self-fertilization because selected genes are more likely to be represented in both the male pollen and the female ovules if they come from the same plant (Clarke and Merlin, 2016). Moon *et al*. (2010) developed a production method for feminized seeds by inducing the formation of male flowers and pollen on female plants that can be used for self fertilization of hemp. However, feminized seeds are not suitable for the production of non-fertilized flowers because the greater the generation of feminized seeds, the higher the ratio of monoecious plants (Faux *et al*., 2014). If male plants can produce male and female flowers on the same plant, not only can desirable traits be fixed in an early generation, but also dioeciousness can be maintained. This experiment was conducted to develop a self-fertilization method by the formation of sufficient amounts of female flowers and seeds from male plants of dioecious hemp by the timing of ethephon treatment.

Materials and Methods

Plant material and growth condition

Hemp seeds of the Korean landrace, a dioecious cultivar, were sown in a tray pot with 72 cells, and the seedlings were planted in Wagner pots (size: 1/2,000 a), 15 days after sowing. The Wagner pots were filled with nursery media with constituents of 4%, 7%, 68%, 14.7%, 0.2%, and 0.06% of zeolite, perlite, coconut peat, peat moss, fertilizer, and wetting agent, respectively. The plants were grown in Walk In Chamber with a size of 3,150 ㎜, 5,800 ㎜, and 3,050 ㎜ in width, length, and height, respectively. The environment conditions of the Walk In Chamber were adjusted to 25℃, 65%, 144 ㎛ol m^{-2} s^{-1}, and 500 ㎎ kg^{-1} in temperature, humidity, luminous intensity, and concentrations of CO_2, respectively. To induce early flowering, the Walk In Chamber was adjusted to a short day length with light and dark periods of 12 hours. Twenty days after planting, the sex of hemp plants was identified by the appearance of primordia at the node, and male plants were used as the material for the experiment. Ten female plants of the same cultivar were grown as controls.

Treatment of ethephon and investigation of growth characteristics on plants and seeds

The sex of hemp plants cannot be distinguished in the vegetative stage. In conditions of short day length, the plants initiate sex differentiation with the formation of primordia (Mediavilla *et al*., 1998; Moliterni *et al*., 2004). Male primordia can be identified by their curved claw shape, soon followed by the differentiation of round pointed flower buds as shown in Fig. 1 (Mediavilla *et al*., 1998). Sakthinathan *et al*. (2017) showed that spraying 500 ㎎ L^{-1} to *Cucurbita maxima* was effective to increase the number of female flowers per plant without any side effect including decrease of seed germination. Thus, ethephon 39% (NongHyup Chemical, Seongnam, Korea) was diluted to 500 ㎎ L^{-1} with water. The ethephon solution was sprayed onto plants at soon after primordia formation, and subsequently, seven and fourteen days later (Fig. 1).

The plant ratio of female flower formation and growth characteristics, including stem height, were investigated 15 and 45 days after treatment with ethephon solution. The

Fig. 1. Male plants at the time of ethephon treatment after male primordia formation, soon after (A), 7 days later (B) and 14 days later (C). At the time of primordia formation, primordia were formed between stipule and petiole under shoot tip. At time goes on, leaves became smaller, decreased and more flowers were formed.

characteristics of seeds and germination rate were investigated using harvested seeds. The plant ratio of female flower formation was measured with ten plants per replicate. Of the ten plants, we counted the number of plants that formed stigma and calyx, and calculated the percentage. Stem height was measured from the distal end to the apex. The number of branches was counted from the first branch attached to the trunk. The number of fruiting nodes on the branch was counted as seed bearing nodes on the longest branch.

The characteristics of seeds including the number of harvested seeds were investigated after harvest. The number of harvested seeds, weight of 1,000 grains, seed size, and germination rate were measured with seeds from 6 plants in each treatment. Individual plants were regarded as replications for statistical analysis. The size, length, and width of the seeds were measured by copying the seeds with a document copier (FX DocuCentre-III 3007 PCL 6, Tokyo/Japan, Fuji Xerox) and measuring the photocopied 20 seeds per replication (Moon *et al*., 2020).

Statistical analysis

Duncan multiple range test was used to determine significant differences between the timing of ethephon treatment and control. Statistical analyses were carried out using the SAS statistical package (version 9.3-2012; SAS Institute, Cary, USA).

Results and Discussion

By treatment of ethephon to male plants of hemp, female flowers were formed on the shoot and branch even if the flowering amounts were different by treatment timing. The plant ratio of female flower formation was 100% in the ethephon treatment at soon after primordia formation, which was the same as that in the control of normal female plants, but decreased to 40% and 0%, at seven and fourteen days after primordia formation, respectively (Table 1). Ethephon is converted into enthylene upon metabolism by plants and exerts a potency of plant growth regulator (Potter *et al*., 1990; Sakthinathan *et al*., 2017). Ethylene has a feminization effect on the sex expression of hemp (Galoch, 1978; Moliterni *et al*., 2004). As the treatment of ethephon were undertaken earlier, the effect of female flower formation was greater. Stem height showed statistical significance at the 5% level among the treatment and control groups as 141㎝, 158 ㎝, 178 ㎝ and 122 ㎝ in ethephon treatment at soon, seven, fourteen days after primordia formation and control of normal female plants, respectively. However, the number of branches did not show statistical significance at the 5% level among the treatment and control groups at 26, 24, 26, and 25 in ethephon treatment at soon, seven and fourteen days after primordia formation, and control of normal female plants, respectively (Table 1). Ethylene not only accelerates the ripeness of grains or fruits but also the growth inhibition of plants (Aryal and

Table 1. Plants ratio of female flowers formation and growth characteristics by timing of ethephon treatment on male hemp plants

Timing of ethephon treatment	Plants ratio of female flowers formation (%)	Stem height (㎝)	Number of branches
Soon after primordia formation	100 a[z]	141 c	26 a
Seven days after primordia formation	40 b	158 b	24 a
Fourteen days after primordia formation	0 c	178 a	26 a
Female plants (Control)	100 a	122 d	25 a

[z]Mean separation within columns by Duncan's multiple range test at $p = 0.05$.

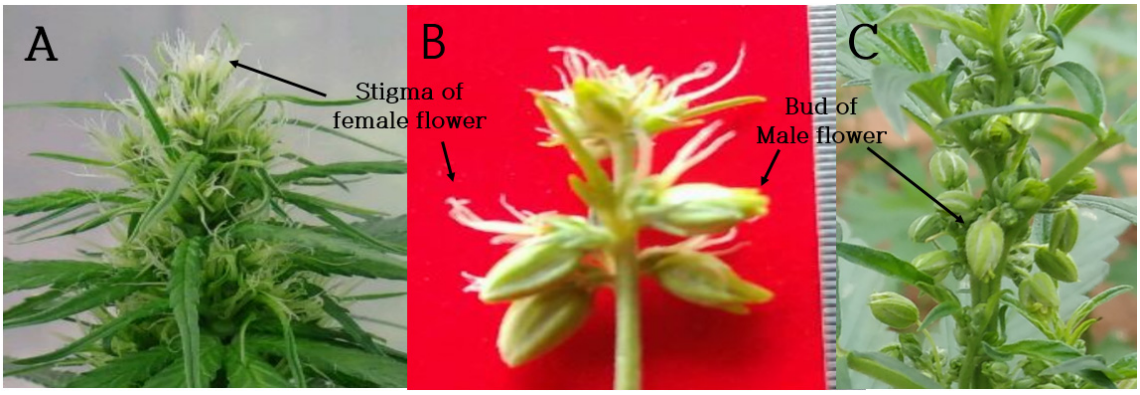

Fig. 2. Shape of flowers and reproductive organs of hemp plants. Female flowers on female plant (A) and male and female flowers induced by ethephon treatment to male plant (B), and buds of male flowers on normal male plant (C). In Figure B, each marking on the ruler is 0.5 ㎜.

Ming, 2014; Dubois *et al*., 2018; Scott and Leopold, 1967). In this experiment, the shorter stem height in the treatment of ethephon was thought to result from the effect of growth inhibition in the hemp plants.

Female and male flowers were formed side by side on male plants which were treated by ethephon whereas normal female and male plant formed female and male flowers exclusively, respectively (Fig. 2). Male inflorescence of hemp are hanged to panicle sometimes branched, and female inflorescence are raceme developing densely at the apex of the plant or at the axils of leaves or lateral branches (Moliterni *et al*., 2004). In this experiment, female and male flowers were hanged to panicle sometimes branched like a normal male inflorescence.

In a review by Hall *et al*. (2012) on inflorescence of hemp, female flowers consisted of a single ovary, bract, and dual stigmas. However, female flowers of male plants had 5 stigmas in contrast to the dual stigma of the flowers of female plants in this experiment (Fig. 3).

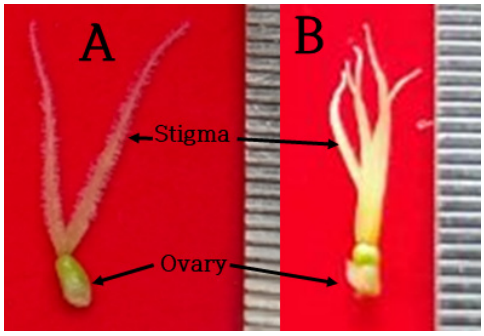

Fig. 3. Morphological comparison of female reproductive organs between female (A) and male (B) plants. In the Figure, each marking on the ruler is 0.5 ㎜.

In male plants which were treated by ethephon, smaller seeds were formed loosely on the node, whereas female plants formed larger seeds densely (Fig. 3). The number of fruiting nodes on the branches were 17, 9, 0, and 24 in ethephon treatment at soon, seven, fourteen days after primordia formation and control of normal female plants, respectively,

Table 2. Fruiting characteristics of hemp plants by timing of ethephon treatment on male hemp plants

Timing of ethephon treatment	Number of fruiting nodes on branch	Number of harvested seeds (grains/plant)
Soon after primordia formation	17 b[z]	960 b
Seven days after primordia formation	9 c	183 c
Fourteen days after primordia formation	0 d	0 d
Female plants (Control)	24 a	1,404 a

[z]Mean separation within columns by Duncan's multiple range test at p = 0.05.

Fig. 4. Seed formation of female (A), male plants (B) and size comparison between seeds from male and female plants (C). In Figure C, each marking on the ruler is 0.5 ㎜.

Table 3. Characteristics of harvested seeds and germination rate by timing of ethephon treatment on male hemp plants

Timing of ethephon treatment	Weight of 1,000 grains (g)[a]	Seed size (㎜)		Germination rate (%)
		Length	Width	
Soon after primordia formation	7.3 b[z]	3.34 b	2.67 b	53 b
Seven days after primordia formation	5.1 c	2.89 c	2.27 c	54 b
Female plants (Control)	20.6 a	4.27 a	3.36 a	80 a

[z]Mean separation within columns by Duncan's multiple range test at p = 0.05.

which were statistically significant at the 5% level (Table 2). Number of harvested seeds were also showed statistical significance at 5% level among the treatments and control as 960, 183, 0 and 1,044 in ethephon treatment at soon, seven, fourteen days after primordia formation and control of normal female plants, respectively (Table 2). As mentioned above, ethylene has a feminization effect on the sex expression of hemp. Plant hormones, including ethylene, have more effects when treated at the early growth stage (Bandara *et al*., 1998). As the treatment of ethephon was undertaken earlier, the number of fruiting nodes on the branch was greater. Further, with a greater number of fruiting nodes, more seeds were harvested in this experiment.

In the characteristics of harvested seeds, weight of 1,000 grains were 7.3 g, 5.1 g and 20.6 g in the ethephon treatment at soon, seven, fourteen days after primordia formation and control of normal female plants, respectively. Seed size, examined by length and width, showed statistical difference at 5% level among the treatments and control as 3.34 and 2.67 ㎜, 2.89 and 2.27 ㎜, 4.27 ㎜ and 3.36 ㎜ in ethephon treatment at soon, seven, fourteen days after primordia formation and control of normal female plants, respectively. In this experiment, the seeds from male plants were much smaller than normal seeds, which were harvested from female plants (Fig. 4). The seeds from male plants could be germinated and grown to seedlings, although the germination

rate was lower than that of seeds from female plants. Germination rates were statistically different at the 5% level between seeds from male and female plants as 53−54% and 80% in seeds from male and female plants, respectively, but there was no statistically significant difference between the timing of ethephon treatment (Table 3). In conclusion, treating ethephon to male plants at soon after primordia formation will be good strategy in breeding program to breed inbred lines of hemp by self-fertilization.

Acknowledgements

This study was supported by the Research Program for Agriculture Science and Technology Development at the Rural Development Administration of Korea (project number PJ015091022020).

Conflicts of Interest

The authors declare that they have no conflict of interest.

References

Aryal, R. and R. Ming. 2014. Sex determination in flowering plants: papaya as a model system. Plant Sci. 217:56-62.

Bai, Q., Z. Ma, Y. Zhang, S. Su and P. Leng. 2019. The sex expression and sex determining mechanism in *Pistacia* species. Breed. Sci. 69(2) 205-214.

Bandara, M.S., K.K. Tanino and D.R. Waterer. 1998. Effect of pot size and timing of plant growth regulator treatments on growth and tuber yield in greenhouse-grown Norland and Russet Burbank potatoes. J. Plant Growth Regul. 17(2):75-79.

Beutler, J.A., and A.H. Marderosian. 1978. Chemotaxonomy of cannabis I. Crossbreeding between *Cannabis sativa* and *C. ruderalis*, with analysis of cannabinoid content. Econ. Bot. 32(4):387.

Cabezudo, B., M. Recio, J. Sánchez-Laulhé, M.D.M. Trigo, F.J. Toro and F. Polvorinos. 1997. Atmospheric transportation of marihuana pollen from North Africa to the southwest of Europe. Atmos. Environ. 31(20):3323-3328.

Carter, C. 2017. Cannabis plants named 'Katelyn Faith'. USA. Plant Patent. US 20170172040P1.

Chandra, S., H. Lata, M.A. ElSohly, L.A. Walker and D. Potter. 2017. Cannabis cultivation: Methodological issues for obtaining medical-grade product. Epilepsy Behav. 70:302-312.

Clarke, R.C. and M.D. Merlin. 2016. Cannabis domestication, breeding history, present-day genetic diversity, and future prospects. Cr. Rev. Plant Sci. 35(5-6):293-327.

Cohen, Y. 2014. Cannabis plants named 'Erez'. USA. Plant Patent. US 20140245494A1.

_____. 2020. Cannabis plants named 'Avidekel'. USA. Plant Patent. US 20200008336P1.

de Meijer, E.P.M and L.J.M. Van Soest. 1992. The CPRO cannabis germplasm collection. Euphytica 62(3):201-211.

Dubois, M., L. Van den Broeck and D. Inzé. 2018. The pivotal role of ethylene in plant growth. Trends Plant Sci. 23(4):311-323.

Faux, A.M., A. Berhin, N. Dauguet and P. Bertin. 2014. Sex chromosomes and quantitative sex expression in monoecious hemp (*Cannabis sativa* L.). Euphytica 196(2):183-197.

Galoch, E. 1978. The hormonal control of sex differentiation in dioecious plants of hemp (*Cannabis sativa* L.). The influence of plant growth regulators on sex expression in male and female plants. Acta Soc. Bot. Pol. 47(1-2):153-162.

Hall, J., S.P. Bhattarai and D.J. Midmore. 2012. Review of flowering control in industrial hemp. J. Nat. Fibers 9(1):23-36.

Heikrujam, M., K. Sharma, M. Prasad and V. Agrawal. 2015. Review on different mechanisms of sex determination and sex-linked molecular markers in dioecious crops: a current update. Euphytica 201(2):161-194.

Holmes, D. 2019. Cannabis plants named 'DD-CT-BR5'. USA. Plant Patent. US00PP30668P3.

Kim, S.K. 2019. The Use situation of cannabis and its value as a resource plants. *In* Proceedings of the Plant Resources Society of Korea Conference. p. 6.

Lewis, M.A. 2020. Cannabis plants named 'Lemon Crush OG'. USA. Plant Patent. US 00PP31535P3.

Mahlberg, P.G. and E.S. Kim. 2004. Accumulation of cannabinoids in glandular trichomes of cannabis (*Cannabaceae*). J. Indust. Hemp 9(1):15-36.

Mandolino, G., A. Carboni, S. Forapani, V. Faeti and P. Ranalli. 1999. Identification of DNA markers linked to the male sex in dioecious hemp (*Cannabis sativa* L.). Theor. Appl. Genet. 98(1):86-92.

McPartland, J.M. 2018. Cannabis systematics at the levels of family, genus, and species. Cannabis Cannabinoid Res. 3(1): 203-212.

Mediavilla, V., M. Jonquera, I. Schmid-Slembrouck and A. Soldati. 1998. Decimal code for growth stages of hemp (*Cannabis sativa* L.). J. Int. Hemp Ass. 5(2):65, 68-74.

Moliterni, V.C., L. Cattivelli, P. Ranalli and G. Mandolino. 2004. The sexual differentiation of *Cannabis sativa* L.: A morphological and molecular study. Euphytica 140(1-2):95-106.

Moon, Y.H., B.C. Koo, Y.H. Choi, S.T. Bark, S.H. Ahn, Y.L. Cha and S.J. Suh. 2010. Seed production by induction of male flowers on female plants of hemp (*Cannabis sativa* L.). Korean J. Crop Sci. 55(4):327-332 (in Korean).

Moon, Y.H., Y.S. Song, B.C. Jeong and J.G. Bang. 2005. Variation on fatty acid profile including γ-linolenic acid among hemp (*Cannabis sativa* L.) accessions. Korean J. Medicinal Crop Sci. 13(4):190-193 (in Korean).

_____. 2006. Cluster analysis and growth characteristics of hemp (*Cannabis sativa* L.) germplasm. Korean J. Crop Sc. 51(5):483-490 (in Korean).

Moon, Y.H., Y.L. Cha, J.E. Lee, K.S. Kim, D.E. Kwon and Y.K. Kang. 2020. Investigation of suitable seed sizes, segregation of ripe seeds, and improved germination rate for the commercial production of hemp sprouts (*Cannabis sativa* L.). J. Sci. Food Agr. 100(7):2819-2827.

Potter, D.A., M.C. Buxton, C.T. Redmond, C.G. Patterson and A.J. Powell. 1990. Toxicity of pesticides to earthworms (*Oligochaeta: Lumbricidae*) and effect on thatch degradation in Kentucky bluegrass turf. J. Econ. Entom. 83(6):2362-2369.

Sakthinathan, B., V. Swaminathan, P. Balasubramanian and T. Sivakumar. 2017. Effect of ethrel on sex expression on pumpkin (*Cucurbita moschata* L.). Int. J. Chem. Stud. 5(6): 964-966.

Scott, P.C. and A.C. Leopold. 1967. Opposing effects of gibberellin and ethylene. Plant Physiol. 42(7):1021.

Small, E. 2015. Evolution and classification of *Cannabis sativa* (marijuana, hemp) in relation to human utilization. Bot. Rev. 81(3):189-294.

Spitzer-Rimon, B., S. Duchin, N. Bernstein and R. Kamenetsky. 2019. Architecture and florogenesis in female *Cannabis sativa* plants. Front. Plant Sci. 10:350.

Zager, J.J., I. Lange, N. Srividya, A. Smith and B.M. Lange. 2019. Gene networks underlying cannabinoid and terpenoid accumulation in cannabis. Plant Physiol. 180(4):1877-1897.

(Received 13 October 2020 ; Revised 12 November 2020 ; Accepted 23 November 2020)