



Influence of Copper Sulphate on Anther Culture for Haploid Production in African Marigold (*Tagetes erecta* L.)

Uzma Mehraj^{a,b}, Kanwar Pal Singh^{a*}, Gunjeet Kumar^a and Sapna Panwar^a

^a *Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi, 110012, India.*

^b *ICAR-Directorate of Floriculture, Pune, 411036, India.*

Authors' contributions

This work was carried out in collaboration among all authors. Author KPS did the conceptualization of research. Authors KPS, UM, GK, and SP designed the experiments. Author UM executed field/lab experiments and data collection and Analysis of data and interpretation. Authors KPS, UM, GK and SP prepared the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJECC/2024/v14i13821

Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/111763>

Original Research Article

Received: 28/10/2023

Accepted: 04/01/2024

Published: 07/01/2024

ABSTRACT

The present study was carried out to improve the yield of androgenesis in marigold, for which copper sulfate was tested during the pretreatment of anther culture at various concentrations. The best results were obtained when copper sulfate was added at 15 μ M and 20 μ M. With the addition of copper sulphate at 20 μ M, the percent of responding anthers increased from 81.6 to 92.2 %. While percent caulogenesis increased from 66.8 to 78.4 % with copper sulphate concentration of 15 μ M. With the same concentration of copper sulphate (15 μ M), the number of shoot buds per anther increased from 7.4 to 9.6 and the number of regenerants per anther increased from 5.8 to 8.6. The positive influence of copper sulphate may be due to an increase in the microspore survival during anther culture.

*Corresponding author: E-mail: kanwariari@gmail.com;

Keywords: Anther culture; copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$); haploids.

1. INTRODUCTION

“Marigold (*Tagetes* spp.) is one of the economically important ornamental crops grown worldwide. It belongs to the family Asteraceae and is native to South and Central America (Mexico). It is one of the most important commercial loose flower crops grown in India and ranks first amongst loose flower crops in area and production. There are about 33 species of the genus *Tagetes*, out of which, *Tagetes erecta* L. (Aztec or African marigold) and *Tagetes patula* L. (French marigold) are highly important for loose flower production. In India, marigold is being cultivated in an area of 55.89 thousand hectares with the production of 511.31 thousand million tons of loose flowers and 4.25 thousand million tons of cut flowers and is almost grown throughout the country” [1,2]. The major marigold growing states are Karnataka, Gujarat, Maharashtra, Haryana, Andhra Pradesh, Uttar Pradesh, etc. Haploids are plants that contain a gametic chromosome number (n). They can originate spontaneously in nature or because of various induction techniques. Doubled haploids are produced by the process of chromosome doubling of the haploids. Although haploidy was identified much earlier [3] and attempted in commercial crop improvement [4], it was not until the work of Guha and Maheshwari [5], Nitsch and Nitsch [6] and Kasha and Kao [7] that the potential of anther culture and wide hybridizations to create haploid plants revived plant breeders interest. “The production of pure lines using doubled haploids has several advantages over conventional methods. Using doubled haploid production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. Hence the time saving is substantial. For self-incompatible species, dioecious species and species that suffer from inbreeding depression due to self-pollination, haploidy may be the only way to develop inbred lines” [8]. Furthermore the genetic upgradation of crops through conventional breeding approaches requires longer time so there is a need to assist breeding programs following certain biotechnological tools *i.e.*, induction of doubled haploids and their use in breeding to shorten the breeding cycle. It has been widely used in breeding programmes of many crop plant species. *In vitro* production of doubled haploids has been successfully done in crops like tall fescue [9], sugar beet [10], African violet [11],

Pelargonium roseum [12], *Lilium davidii* var. *Willmottiae* [13], *Lilium longiflorum* [14], *Narcissus tazetta* [15] etc.

“In various cereals like barley, the use of androgenesis has led to the generation of several cultivars that are currently cultivated in many countries. However, several lines remain recalcitrant for microspore embryogenesis, mostly because of genotypic reasons” [16] “Also, there is the problem of albino plantlet production during the improvement of cultivars through androgenesis” [17]. Hence, optimization of the anther culture protocol remains of considerable interest for plant breeders.

The positive influence of copper during *in vitro* culture of various explants has been reported by several authors in barley [18,19] and other cereals [20]. It has been reported that increasing the amount of copper sulfate in the culture medium increases the yield of plant regeneration from callus cultures [21], allows the production of green plantlets for more than a year from scutellum-derived callus in recalcitrant lines [18], and improves the behavior of polyembryonic cultures of scutellum [19]. We have investigated the possible effect of copper during anther culture in marigold.

2. MATERIALS AND METHODS

African marigold grown in the field was used for the present study. Experiment material consisted of buds with size ranging from of 2 to 2.5cm of which most microspores had reached the mid- or late-uninucleate stage [22]. A total of 12-14 buds were taken. Buds were collected from the field in the morning; and were thoroughly washed with tap water and sterilized by spraying with 70% ethanol and they were then pretreated with 0.3M mannitol solution for 4 days as described previously [23]. The pretreatment medium was supplemented with various concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (5, 10, 15, 20, 25 and 30 μM). After pretreatment, anthers were cultivated according to Kumar et al. [22].

2.1 Experimental Designs

The experiments were laid out in completely randomised design (CRD). Each treatment had 20-30 units and with four replications. Each experiment was repeated at least twice, and the reported data are the means of two

experiments. Wherever applicable the data are presented as mean \pm standard error. The percentage data was subjected to Arc Sin transformation. After transformation of original values, statistical analysis was performed by using ANOVA.

3. RESULTS AND DISCUSSION

“Marigold anther culture technology used in breeding programs is associated with a relatively low yield of pollen-derived embryos and green plant regenerants and high a frequency of albino plant regenerants” [22]. “The same has been reported to occur in wheat and barley” [24]. “Therefore, various modifications have been made to improve this method, particularly regarding effective pretreatment methodology” [25-28]. This investigation showed that supplementation of copper in mannitol at the time of marigold anther pre-treatment increases the percentage of green plant regenerants. In the present study, (as indicated in Table 1), with the addition of copper sulphate at 20 μ M, the percentage of responding anthers increased from 81.6 (with only mannitol) to 92.2 % (with mannitol and copper sulphate), followed by 91.2% with a copper sulphate concentration of 25 μ M. Meanwhile, percent caulogenesis increased from 66.8 to 78.4 % with a copper sulphate concentration of 15 μ M, followed by 78.2% with the addition of copper sulphate in the concentration of 20 μ M. With the same concentration of copper sulphate (15 μ M), the number of shoot buds per anther increased from 7.4 to 9.6 followed by 8.6 with the copper sulphate in concentrations of 20 μ M through 30 μ M and number of regenerants per anther increased from 5.8 to 8.6 with copper sulphate concentration of 15 μ M followed by 8.4 with copper sulphate in the concentrations of 20 μ M and 25 μ M (Fig. 1). No significant differences were found in number of days taken to callus induction and number of days taken to shoot bud induction. Increasing the concentration of copper sulphate from 5 to 20 μ M lead to an increase in the percent of responding anthers, percent caulogenesis, number of shoot buds per anther and number of regenerants per anther while further increase lead to a reduction in the above-mentioned parameters, which may be due to the toxicity caused by the increased concentration of copper sulphate. These results are in accordance with data previously reported in barley considering the

interest of optimizing the copper concentration during pretreatment and in culture media [18,19,21]. “Similar results have been obtained in other studies as well [29], wherein it was reported that adding copper sulfate from 1 to 20 mM during both anther pretreatment and culture globally improved the yield of androgenesis in the barley winter cv. Igri. It was further reported that the anther response increased when copper sulfate was used at concentrations between 5 and 18 mM reaching up to 73.6% at 15 mM. Copper seems to affect the step of the androgenesis in the *in vitro* culture of marigold. The beneficial effect of copper is optimal during pretreatment, which suggests that the physiological events leading to microspore reorientation and green plant regeneration occur during pollen development or during the earliest steps of androgenesis” [17].

“Copper sulfate addition has been shown to improve the behavior of barley microspores during androgenesis, increasing their survival during the whole process. The deficiency of copper is known to drastically affect plant reproduction” [30,31]. The lack of copper in the anther of cereals changes tapetum physiology causing cell hypertrophy [32,33] and modifications of RNA metabolism [34], which results in disturbances of nucleus metabolism in the microspore and reduction of pollen fertility [34,35]. Therefore, performing anther culture in marigold, the increase of anther response in the presence of high copper sulfate concentrations is in accordance with previous data and confirms the beneficial influence of appropriate concentrations of copper on pollen physiology.

“In cereals, it is reported that copper plays an important role in the anther during pollen development as it affects both tapetum and pollen metabolism” [32,34]. “Copper deficiency induces tapetum dysfunctioning whereas pollen undergoes abnormal polyploidy and inhibition of DNA synthesis. In several cases, copper deficiency has led to pollen abortion and male sterility. Moreover, copper is involved in many other physiological processes like chlorophyll synthesis and photosynthesis” [36]. Copper has a beneficial influence on regeneration during *in vitro* culture of plants and it is important during both pollen development and plant physiology. Copper also has a major influence on *in vitro* plant physiology [18,19,21].

Table 1. Effect of copper sulfate on haploid production in marigold *via* anther culture

Treatment(s)	Percent of responding anthers (%)	Days taken to callusing	Percent caulogenesis (%)	Days to shoot bud induction	No. of shoot buds per anther	No. of regenerants per anther
T ₀ (Control)	81.600 (64.584)	11.600	66.800 (54.798)	15.400	7.400	5.800
T ₁ (CuSO ₄ . 5H ₂ O @ 5 µM)	81.800 (64.726)	11.600	66.600 (54.675)	15.400	7.400	5.600
T ₂ (CuSO ₄ . 5H ₂ O @ 10 µM)	82.000 (64.876)	11.600	67.000 (54.917)	15.400	7.400	5.600
T ₃ (CuSO ₄ . 5H ₂ O @ 15 µM)	82.400 (65.184)	11.800	78.400 (62.286)	15.600	9.600	8.600
T ₄ (CuSO ₄ . 5H ₂ O @ 20 µM)	92.200 (73.770)	11.800	78.200 (62.145)	15.400	8.600	8.400
T ₅ (CuSO ₄ . 5H ₂ O @ 25 µM)	91.200 (72.729)	11.600	77.800 (61.868)	15.400	8.600	8.400
T ₆ (CuSO ₄ . 5H ₂ O @ 30 µM)	91.000 (72.523)	11.400	78.000 (62.005)	15.400	8.600	7.800
±SE(m)	0.469	0.233	0.438	0.245	0.245	0.288
C.D. (P≤ 0.05)	1.366	N/A	1.274	N/A	0.713	0.838

*Values in parenthesis are angular values

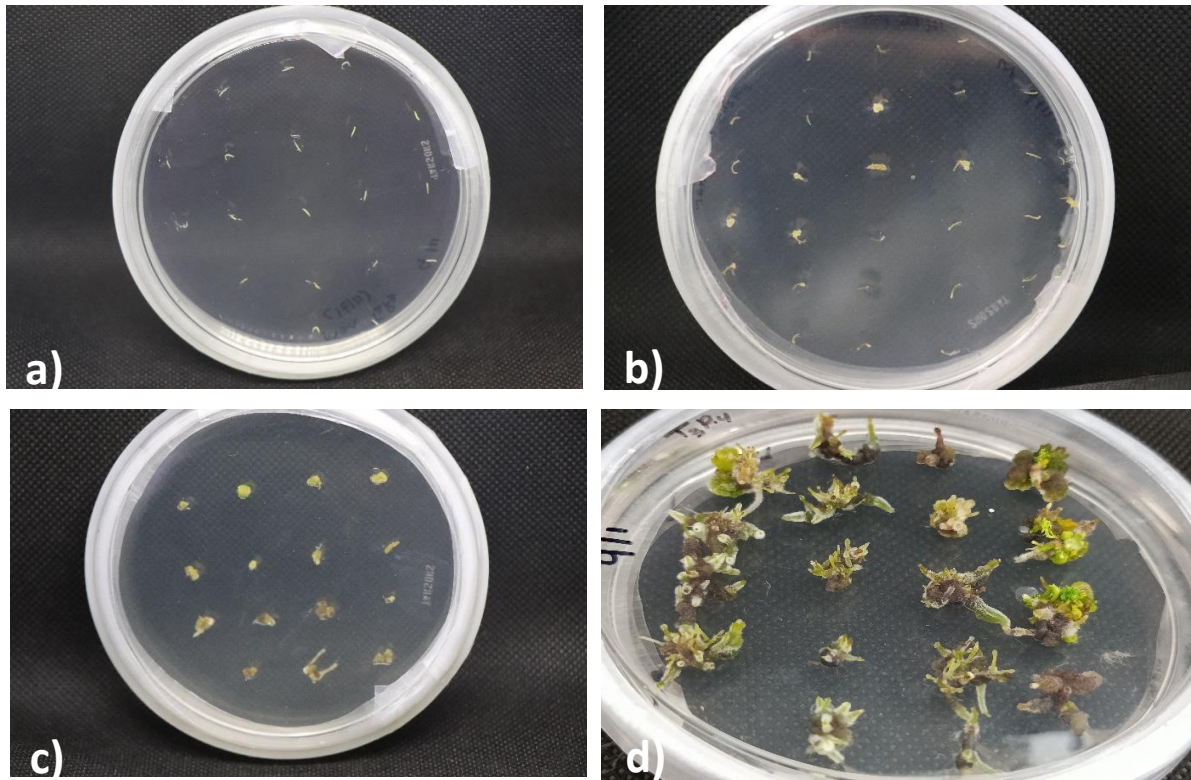


Fig. 1. a) inoculation of anthers with copper sulfate added to the pretreatment solution @ 20 μ M. b) Swelling of anthers c) Callus induction in the anthers d) Shoot induction from the anthers

“Copper deficiency induces chlorosis in leaves, and results in decrease of chlorophyll content” [37]. “Previous investigations have shown that, although plants accumulate copper only in small amounts, this element has great importance in plant metabolism. In anther culture, copper deficiency is associated with an increased formation of albino plants” [24]. Several other observations regarding the role of copper in cereal anther physiology are available [32,34], but poor information is available regarding the effect of copper on androgenesis in flower crops.

4. CONCLUSION

Copper has a positive influence on obtaining haploid plants by the anther culture as it leads to the reduction of the number of albino plants and increases the number of green plant-regenerants. These effects ultimately lead to improved survival of microspores during tissue culture stages and cause the synchronisation of the first microspore symmetric division [32,29,24]. Our studies and results obtained were in agreement with the above statement.

ACKNOWLEDGEMENT

The first author is thankful to the department of science and technology, New Delhi for the financial assistance in the form of Junior Research Fellowship.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rydberg PA. *Tagetes*, Flora of North America. 1915;34:148-159.
2. Anonymous. Indian horticulture database published by National Horticulture Board. Ministry of Agriculture and Farmers Welfare; 2014. Available:www.nhb.gov.in
3. Blakeslee AF, Belling J, Farnham ME, Bergner AD. A haploid mutant in the Jimson weed, *Datura stramonium*. *Science*. 1922;55:646-647.

4. Chase SS. Monoploids and monoploid derivatives of maize. The Botanical Review. 1960;35:117-167.
5. Guha S and Maheshwari SC. *In vitro* production of embryos from anthers of Datura. Nature. 1964;204:497.
6. Nitsch JP, Nitsch C. Haploid plants from pollen grains. Science. 1969;163:85-87.
7. Kasha KJ, Kao KN. High frequency haploid production in barley (*Hordeum vulgare* L.). Nature. 1970;225:874-876.
8. Jana Murovec, Borut Bohanec. Haploids and doubled haploids in plant breeding, plant breeding, Ibrokhim Y. Abdurakhmonov, Intech Open; 2012.
9. Kasperbauer MJ, Eizenga GC. Tall fescue doubled haploids via tissue culture and plant regeneration. Crop Science. 1985;25:1091-95.
10. Klimek-Chodacka M, Baranski R. Comparison of haploid and doubled haploid sugar beet clones in their ability to micro-propagate and regenerate. Electronic Journal of Biotechnology. 2013; 16(2):1-10.
11. Hughes KW, Bell SL, Caponetti JD. Anther derived haploids of the African violet. Canadian Journal of Botany, 1975;53(14): 1442-1444.
12. Kato M, Suga T, Tokumasu S. Effect of 2, 4-D and NAA on callus formation and haploid production in anther culture of *Pelargonium roseum*. Memoirs of the College of Agriculture, Ehime University. 1980;24 (2):199-207.
13. Gu ZP, Cheng KC. Studies on induction of pollen plantlets from the anther cultures of lily. Acta Botanica Sinica. 1982;24:28-32.
14. Qu Y, Mok MC, Mok DW, Stang JR. Phenotypic and cytological variation among plants derived from anther cultures of *Lilium longiflorum*. *In vitro* Cellular and Developmental Biology. 1988;24(5):471-476.
15. Chen L, Zhu X, Gu L, Wu J. Efficient callus induction and plant regeneration from anther of Chinese narcissus (*Narcissus tazetta* L. var. *Chinensis* Roem). Plant Cell Reports. 2005;24(7):401-407.
16. Larsen ET, Tuvevsson IKD, Andersen SB. Nuclear genes affecting percentage of green plants in barley (*Hordeum vulgare* L.) anther culture. Theoretical and Applied Genetics. 1991;82:417-420.
17. Caredda S, Doncoeur C, Devaux P, Sangwan RS, Clement C. Plastid differentiation during androgenesis in albino and nonalbino producing cultivars of barley (*Hordeum vulgare* L.), Sexual Plant Reproduction. 2000;13:95-104.
18. Cho MJ, Jiang W and Lemaux PG. Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism, Plant Science. 1998;138:229-244.
19. Nuutila AM, Hamalainen J, Mannonen L. Optimization of media nitrogen and copper concentrations for regeneration of green plants from polyembryogenic cultures of barley (*Hordeum vulgare* L.). Plant Science. 2000;151:85-92.
20. Guo YD, Pulli S. An efficient androgenic embryogenesis and plant regeneration method through isolated microspore culture in timothy (*Phleum pratense* L.). Plant Cell Reproduction. 2000;19:761-767.
21. Dahleen. Improved plant regeneration from barley callus cultures by increased copper levels, Plant Cell, Tissue and Organ Culture. 1995;43:267-269.
22. Kumar KR, Singh KP, Bhatia R. Optimising protocol for successful development of haploids in marigold (*Tagetes* spp.) through *in vitro* androgenesis. Plant Cell, Tissue and Organ Culture. 2019;138(1):11-28.
23. Mehraj U, Singh KP, Kumar G and Panwar S. Influence of anther pretreatment on the efficiency of haploid production in marigold (*Tagetes erecta* L.), AMA, Agricultural Mechanization in Asia, Africa and Latin America, 2022;54(11):16287-16295.
24. Jacquard C, Nolin F, Hécart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan RS, Devaux P, Mazeyrat-Gourbeyre F, Clément C. Microspore embryogenesis and programmed cell death in barley: Effects of copper on albinism in recalcitrant cultivars. Plant Cell Reports. 2009;28: 1329-1339.
25. Touraev A, Vicente O, Heberle-Bors E. Initiation of microspore embryogenesis by stress. Trends Plant Science. 1997;2(8): 297-302.
26. Hu T, Kasha KJ. A cytological study of pretreatments used to improve isolated microspore cultures of wheat (*Triticum aestivum* L.) cv. Chris. Genome. 1999;42 (3):432-441.
27. Xynias IN, Zamani IA, Gouli-Vavdinoudi E and Roupakias DG. Effect of cold pretreatment and incubation temperature on bread wheat (*Triticum aestivum* L.)

- anther culture. Cereal Research Communications. 2001;29:331-338.
28. Grauda D, Keiða A, Rashal I. Obtaining of doubled haploid lines for Latvian barley and wheat breeding programs by anther culture method. Sordiaretus ja Seemneka-svatus, 2005;9:209-216.
 29. Wojnarowicz G, Jacquard C, Devaux P, Sangwan RS and Clement C. Influence of copper sulfate on anther culture in barley (*Hordeum vulgare* L.). Plant Science 2002; 162:843-847.
 30. Adams P, Graves CJ, Winsor GW. Some effects of copper and boron deficiencies on the growth and flowering of *Chrysanthemum morifolium*, Journal of the Science of Food and Agriculture. 1975;26: 1899-1909.
 31. Bussler W. Physiological functions and utilization of copper, in: J.F. Loneragan, H.D. Robson, R.D. Graham (Eds.), Copper in Soil and Plant, Academic Press, Sydney, Australia. 1981;213-234.
 32. Jewell AW, Murray BG, Alloway BG. Light and electron microscope studies on pollen development in barley (*Hordeum vulgare* L.) grown under copper sufficient and deficient conditions. Plant Cell Environment. 1988;11:273-281.
 33. Alloway BJ, Jewell AW and Murray BG. Effects of subclinical copper deficiency on pollen development and yield cereals, in: P. Morard (Ed.), Proceedings of the Second International Symposium on Role of Micronutrients in Agriculture, Toulouse, France. 1986;31-40.
 34. Azouaou Z, Souvre A. Effects of copper deficiency on pollen fertility and nucleic acids in the durum wheat anther, Sexual Plant Reproduction. 1993;6:199-204.
 35. Graham RD. Male sterility in wheat plants deficient in copper, Nature. 1975;254:514-515.
 36. Maksymiec W. Effect of copper on cellular processes in higher plants, Photosynthesis 1997;34:321-342.
 37. Deriu D, Calace N, Bianca M, Pietroletti M. Morphological and physiological responses of barley plants grown on soils characterized by metal toxicity and metal deficiency. Annales de chimie. 2007;97: 153-162.

© 2024 Mehraj et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:

The peer review history for this paper can be accessed here:
<https://www.sdiarticle5.com/review-history/111763>