Influence of copper sulphate on anther culture for haploid production in African marigold (*Tagetes erecta* L.)

Abstract

In order to improve the yield of androgenesis in marigold, 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 µM and 20 µM. With the addition of copper sulphate at 20µM, 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 µM. with the same concentration of copper sulphate (15 µM), 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 sulfate may be due to an increase in microspore survival during anther culture.

Key words Anther culture, copper sulfate ($CuSO_4 .5H_2O$),haploids.

Introduction

Marigold (Tagetes spp.) is one of the economic 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 [1], 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 [2] and is almost grown throughout the country. The major marigold growing states are Karnataka, Gujarat, Maharashtra, Haryana, Andhra Pradesh, Uttar Pradesh, Orissa 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 (DH's) are produced by the process of chromosome doubling of the haploids. Though haploidy was identified much earlier [3] and attempted in commercial crop improvement [4], it was not until the work of [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 DH 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]. Further the genetic upgradation of crops through conventional breeding approaches requires longer time so there is a need to assist breeding programmes 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 which are currentlycultivated 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 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 the increase of copper sulfate in the culture medium increases the yield of plant regeneration from callus cultures [21], allows the production of green plantlets during more than 1 year from scutellum-derived callus in recalcitrant lines [18], and improves the behavior of polyembryonic cultures of scutellum [19]. In this paper we have investigated the possible effect of copper during anther culture in marigold.

Materials and methods

African marigold variety Local orange grown in the field was used for the present study. Experiments involved buds in the size range of 2-2.5cm in which most microspores had reached the mid- or late-uninucleate stage [22]. 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% ethanoland were pretreated 0.3M mannitol solution for 4 days as described previously [23].The pretreatment medium was supplemented with various concentrations of $CuSO_4.5H_2O$ (5, 10, 15, 20, 25 and 30µM).After pretreatment, anthers were cultivated according to Kumar *et al*. [22].

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 ± standard error. The percentage data was subjected to Arc Sin transformation. After transformation of original values, statistical analysis was performed by using ANOVA.

Results and discussion

Marigold anther culture technology used in breeding programmes is associated with relatively low yield of pollen-derived embryos and green plants-regenerants and high frequency of albino plants-regenerants [22]. 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 [25,26,27,28]. This investigation showed that supplementation of copper in mannitol at time of marigold anther pre-treatment increases the percentage of green plants-regenerants. In the present study, (as indicated in Table I), with the addition of copper sulphate at 20µM, the percent 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 µM. While, percent caulogenesis increased from 66.8 to 78.4 % with copper sulphate concentration of 15 µM, followed by 78.2% with the addition of copper sulphate in the concentration of 20 µM. With the same concentration of copper sulphate (15 µM), 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 µM through 30 µMand number of regenerants per anther increased from 5.8 to 8.6 with copper sulphate concentration of 15 µM followed by 8.4 with copper sulphate in the concentrations of 20 µM and 25 µM (Fig I).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 µM 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 futher increase lead to the reduction in above mentioned parameters which may be due to toxicity caused by 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 by [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. They 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 two parameters of the *in vitro* culture: the step of the androgenesis and the concentration used. The beneficial effect of copper is optimal during pretreatment, which suggest 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 confirm 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 deficiencies 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 and copper has a major influence on *in vitro* plant physiology [18, 19, 21].

Copper deficiency induces chlorosis in leaves, and results in decrease of chlorophyll content [37]. Previous investigations have showed 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 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.

CONCLUSION

Copper has a positive influence on obtaining DH 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 to the above statement.

Table I: Effect of Copper sulfate on doubled 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 ($CuSO_4$. $5H_2O$ @ 5 µM)	81.800 (64.726)	11.600	66.600 (54.675)	15.400	7.400	5.600
T_2 ($CuSO_4$. $5H_2O$ @ 10 µM)	82.000 (64.876)	11.600	67.000 (54.917)	15.400	7.400	5.600

T_3 ($CuSO_4$. $5H_2O$ @ 15 µM)	82.400 (65.184)	11.800	78.400 (62.286)	15.600	9.600	8.600
T_4 ($CuSO_4$. $5H_2O$ @ 20 µM)	92.200 (73.770)	11.800	78.200 (62.145)	15.400	8.600	8.400
T_5 ($CuSO_4$. $5H_2O$ @ 25 µM)	91.200 (72.729)	11.600	77.800 (61.868)	15.400	8.600	8.400
T_6 ($CuSO_4$. $5H_2O$ @ 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

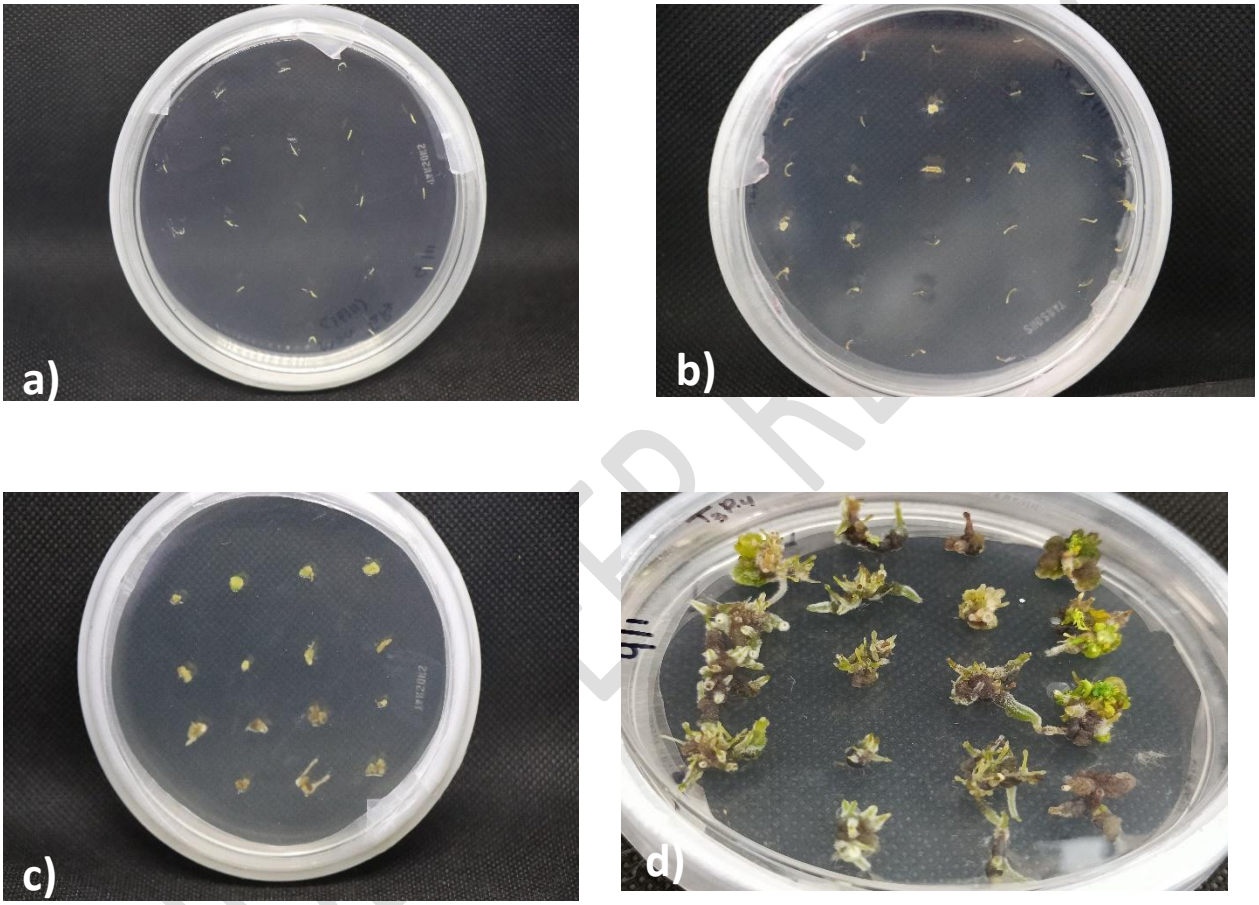

Fig I: a) inoculation of anthers with copper sulfate pretreatment at 20 µM. b) Swelling of anthers c) Callus induction. d) Shoot induction

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