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Effect of Silver Nitrate (AgNO3) and Copper Sulphate (CuSO4) on Callus Formation and Plant Regeneration from Tow Pepper Varieties (Chile Ancho and Misraty) *in Vitro*

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A B S T R A C T

This study was conducted at the Libyan center for biotechnology research laboratories in order to study the effect of different concentrations of silver nitrate and copper sulfate on the callus formation and plant regeneration of two varieties of pepper plant (Misraty and Chile Ancho). Silver nitrate (AgNO3) was added at a concentration of 10, 20, 30, 40 and 50 µM/L /L in the culture media, while copper sulphate (CuSO4) was added at concentrations of 0.1 (concentration of copper sulfate in MS standard medium), 0.5, 1, 2, 3 and 4 µM/L with presence of 17.8 µM/L of Kin and 1.7 µM/L of NAA. The results indicated that adding silver nitrate to the culture medium did not have a positive effect on the rate of callus formation, while it increased the number of plant growths, especially at a concentration of 30 µM/L .As for copper sulfate, the results showed that, for the plant growth parameter there were no significant differences among the treatments of 2.0, 3.0 and 4.0 µM/L compared to the control treatment in Chile Ancho variety, while the number of seedlings and leaves of the obtained plants improved by increasing the concentration of copper sulfate to record the best average of 4.5 and 7.2, respectively, at a concentration of 3 µM/L . Furthermore for the Misraty variety, the results proved that addition of copper sulfate did not have a positive effect on the rate of callus formation, but it effectively affected the plant growth parameter the percentage of plant growth increased from 15% in the control treatment to 45% in treatment of 3 µM/L. The results showed also that the number of leaves increased by increasing the concentration of copper sulfate by recording average of 7.5 leaves at the treatment of 3 µM/L.

1 Introduction

All peppers belong to the genus Capsicum of the Solanaceae family. Chili pepper, chili pepper, and sweet pepper are used interchangeably to describe plants and fruits of the genus Capsicum (Dewitt and Bosland, 1993). There are 27 species of pepper, but only five of them are currently domesticated and cultivated. (*C. annuum L. C. chinense Jacquin, C.baccatum L*, C. *frutescens L, C. pubescens*) Barbara Bakersegl has suggested that the first varieties of chilies originated in the distant past of geological times in an area bounded by the mountains of southern Brazil to the east, by Bolivia to the west North-South, Paraguay and Argentina (Dewitt and Bosland, 1993). The pepper genus Capsicum annuum is the most widely cultivated species in the world. These are the main species grown

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in Hungary, Mexico, China, Korea and the East Indies. In Mexico, the term chile (Spanish for chile) refers to all types of hot and sweet peppers, and thus, chili pepper can be used to describe the plants and pods of the capsicum genus Capsicum.

Micropropagation of cells, tissues, and organs is one of the basic applications of biotechnology in many horticultural crops, in addition to its contribution to genetic improvement. The system of reproduction (regeneration) in pepper varieties did not progress as quickly as it occurred in other species of the Solanaceae family, such as potatoes and tobacco, which is considered a model for tissue culture studies, because it has a great ability to reproduce from cells, tissues and organs, while in the case of pepper organs, reproduction It was difficult considering that the reproduction of all these plants was obtained from any plant extract or other tissue (Alejo and Malagone 2001). Capsicum explants are very sensitive to ethylene during their in vitro development, a characteristic that has been related to recalcitrance (Gammoudi *et al*., 2018). In this way, during in vitro culture, pepper explants usually develop chlorosis, abscission of the foliar primordia and loss of vigor (Monteiro *et al*., 2016). One way to mitigate this effect is using inhibitors of the action of ethylene, which also improve morphogenesis by reducing recalcitrance, achieved by promoting cell elongation and division (Monteiro *et al*., 2016). Among the ethylene inhibiting substances added to the culture medium, AgNO3 has been the most used for Capsicum (Gammoudi *et al*., 2018; Ashwani *et al*., 2017). To overcome the rosette bud formation (resistance to elongation), silver nitrate (AgNO3) or phenyl acetic acid (PAA) has been added to the culture media (Gammoudi *et al*., 2018), although the problem still persists in many materials (Monteiro *et al*., 2016). Other authors have successfully used copper (Cu) to promote the elongation of shoots (Joshi *et al.,* 2007).

Additionally, differences in the organ genic response have been reported among different Capsicum species and genotypes. In this way, large differences in regeneration have been reported in several Capsicum species, including C. annuum, C. baccatum and C. chinense (Sanatombi and Sharma 2008; Orli and Nowaczyk, 2015; Valadez-Bustos *et al*., 2009).

The differences found suggest that the variation associated with genotype in Capsicum materials is a limiting factor in the development of widely applicable regeneration protocols in Capsicum. In fact, individualized protocols are required for different genotypes (caldas *et al*., 1990) so, cultivar-specific media formulations have been designed (Dabauza *et al.,* 2001).

This experiment aims to improve the multiplication rates, where the best combination of growth regulators was chosen, which is 17.8 µM/L of Kin with 1.7 µM/L of NAA obtained from previous experiments with the addition of silver nitrate or copper sulfate in several concentrations, as follows:

First: Silver nitrate (AgNO3) at a concentration of 10, 20, 30, 40 and 50 µM/L.

Second: cupric sulfate (CuSO4) (MS standard medium concentration, 0.5, 1.0, 2.0, 3.0 and 4.0 µM/L.

2 Materials and Methods

This study was carried out at the Plant Tissue Culture Laboratory which belongs to the Libyan center of biotechnology research, with the aim of improving the vegetative growth rates of two hot pepper varieties (Misraty and the Chilean Ancho). Plant seeds were obtained from the local market for the Misraty variety while, seeds of Chile Ancho variety were obtained from Autonom de Aguascalientes university (Mexico).

The process of preparation and sterilization of the food environment and surface sterilization of seeds and their cultivation in the food environment were summarized. The basic nutrient medium was prepared (Murashige & Skooog MS media) (Murashinge and Skoog, 1962). and 45 ml of the nutrient medium was distributed in culture vessels (jars) with a capacity of 330 ml and sterilized in a wet sterilizer (Autoclave) over A temperature of 121°C and an atmospheric pressure of 1.04 kg/cm^2 for 15 minutes. The prepared media was kept for 3 days before use in the next steps of the study. The experiment was started with preparation of MS which, prepared by addition of 3% sucrose and 0.7% agar at pH of 5.7.The prepared media sterilized in Autoclave for 15 minutes ,While about 45 ml of MS media placed in special jars.. Prepared media was kept for three days before use.

The seeds were sterilized inside the laminar airflow cabinet using ethanol at concentration of 70% (v/v) for one mint. Then seeds were treated with sodium hypochlorite solution at a concentration of 2.7% (v/v) of commercial Clorox (5.25% Cl2) for 15 minutes. Finally

seeds were washed by double distilled sterilized water three times for 5 minutes each time. Sterilized seeds were planted on the culture media at a rate of 5 seeds per container, then the cultured plants were incubated in the growth chamber under lighting conditions of 24 µM/L / sec-1 / m-2, a lighting period of 16 hours of light / 8 hours of darkness, and a temperature of 25 ± 2 ° C. Observation of seed germination was conducted carefully time to time until appropriate size of cultured plants has been obtained. The percentage of germination, contamination, and the beginning of root growth were calculated. Moreover obtained plants were incubated for six weeks before being use in the following studies and analyses.

The cultured media was prepared using MS media supplemented with growth regulators NAA and K with concentration of 1.7 and 17.8µM/L respectively whereas, silver nitrate and copper sulfate was added as following First: Silver nitrate (AgNO3) at a concentration of 10, 20, 30, 40 and 50 µM/L, Second: cupric sulfate (CuSO4) (standard MS medium concentration, 0.5, 1.0, 2.0, 3.0 and 4.0 µM/L). However cultured tissues were directly transferred to the growth chamber whereas, plant tissue samples were incubated under conditions of 16 hours of light and 8 hours of darkness at 25 °C to encourage vegetative growth and increase the rate of plant growth for a period of (90±2) days with application of sub culture twice after 30 and 60 days on the same culture medium. The percentage of callus formation, the percentage of vegetative growth, the average number of shoots and the number of leaves for each vegetative growth were calculated after (90±2) days of culture. However the current experiments were conducted mainly to compare the concentrations of each compound independently.

The complete random system **(CRD**) was used with 10 replications for data analysis, and when there were significant differences, the averages were isolated using Duncan's multinomial test at the 5% level.

3 Results and discussion

Establishment of free contamination tissue culture considered as one of the most important steps in plant tissue culture technology in order to succeed in the next steps of the study. The result of contamination percentage measurements table (Alejo and malagone, 2001) in this study showed that the contamination rate was low in all the used treatments whereas, in variety of Chile Ancho the contamination rate recorded 8% while, variety of Misraty on contamination was found in all the used treatments.

Table (1): percentage of contamination and seed germination.

Variety	germination (%) after 30 days	Contamination (%)*	No. of days for starting germination**
Misraty	**79%**	**0%**	**12**
Chile Ancho	**95%**	**8%**	**9**

* The percentage of contamination was calculated after 7 days of cultivation.

** The beginning of germination is the exit of the root

percentage of contamination measured after 7days of culture being mentioned before MS media was prepared and supplemented with growth regulators kinetin(K) and Naphthalene acetic acid (NAA) with concentration of 1.7 and 17.8µM/L respectively whereas, silver nitrate was added to the same culture media with concentrations of 0.0, 10, 20, 30, 40, 50 µM/L. The results of this study table (2) showed that the callus production of Chile Ancho plant samples was significantly higher in treatment of control, 30% 40% silver nitrate compared to other used treatments, whereas control treatment gave a percentage of callus production reached to 80% while treatment of 30% and 40% silver nitrate gave 70% and 65% percentage of callus production respectively. Furthermore the results proved that the other used treatments which are 10, 20,

50 gave lower percentage of callus formation and no significant differences were found among those treatments whereas, those treatments gave a percentage of callus between 40 and 45%. Our obtained results explained that the plant samples of Chile Ancho variety were able to produce callus with large quantities by use of MS culture media supplemented with 30% silver nitrate which mean that addition of silver nitrate with exact concentration has a positive effect on callus formation and induce the plant samples to produce callus. For the vegetative growth parameter the results of this study proved that addition of silver nitrate has a positive effect on plant vegetative growth in general which has been proved in several previous studies whereas, our results proved that addition of 30 and 40µM/L silver nitrate gave the best results while those treatments gave vegetative growth with percentage of 43 and 40% respectively followed by control treatment which gave a percentage of 35%. Furthermore the treatments of 30, 40 and control gave a percentage of vegetative growth significantly higher than the other treatments which are 10, 20 and 50µM/L. In the same line the treatments of 30 and 40 µM/L produced the highest number of auxiliary buds those treatments produced 4.7 and 4.9 auxiliary buds for each cultured plant but, there were no significant differences found among the other treatments which are 10 , 20 and 50µM/L these treatments gave the lowest number of auxiliary buds and the treatment of 10 µM/L produced the lowest number of auxiliary buds among all used treatments. However this obtained results proved the positive role of silver nitrate specially when added with concentration of 30 and 40 µM/L . Our results were in full agreement with (Sharma *et al*., 2008) the study found that addition of silver nitrate had a positive effect on growth and flowering of pepper plant variety of *C. frutescens*.

Number of leaves parameter. In fact to evaluate the plant growth in this study number of leaf parameter was measured for all plant samples. The results showed that addition of silver nitrate increase the number of leaves in each culture plant while, the treatments of 30 and 40µM/L gave the highest number of leaves compared to other treatments whereas, these treatments recorded average of 10.4 and 8.9 leaf for each planted plant respectively. Moreover no significant differences have been found among other used treatments while the treatment of control gave the lowest number of leaves this proved the positive effect of silver nitrate addition on leaf formation for used plant samples . On the same line treatment of control recorded the lowest number of leaf which explain the positive role of silver nitrate by inducing plants to form leaves whereas, control.treatment gave number of leaf of 3.4 leaf in average for each plant sample.

Table (2): Effect of silver nitrate (AgNo3) on callus formation, vegetative growth, Auxiliary buds and number of leaves after 90 days of growth (variety of Chile Ancho)

Conc.AgNO3 µM/L	Number of leaves	Auxiliary buds (%)	Vegetative (%)	Callus formation (%)
0.0	**3.4^{d}**	**2.6^{c}**	**80^{a*}**	**35^{ab}**
10	**7.4^{b}**	**1.6^{d}**	**45^{c}**	**11^{c}**
20	**7.3^{b}**	**3.2^{b}**	**40^{c}**	**28^{ab}**
30	**10.4^{a}**	**4.7^{a}**	**70^{ab}**	**43^{a}**
40	**8.9^{ab}**	**4.9^{a}**	**65^{b}**	**40^{a}**
50	**6.6^{bc}**	**3.2^{b}**	**40^{c}**	**20^{bc}**

* Vertically similar letters, there are no significant differences between them using Duncan's multiple range test at the 5% level.

For Maserati variety results table (Bais *et al*., 2000) our obtained results showed that for callus formation the results proved that treatments of 30% silver nitrate and control obtained the best results and the highest quantities were obtained by those two treatments, which recorded 90 and 94% respectively. On the other hand, for vegetative growth the results showed that the treatment of 30% silver nitrate gave the best results followed by 40% and 20% treatment while the results recorded a percentage of 49, 43 and 35% respectively. Furthermore, treatments of control, 10 and 50% gave the lowest levels of vegetative growth and no

significant differences were found among these treatments.

On the other hand, for auxiliary buds formation the results of this study showed that the addition of silver nitrate led to increase the number of auxiliary buds. Moreover the treatment of 30 and 40% gave the best results and they recorded 5.6 and 5.3 buds respectively which prove the positive effect of silver nitrate on plant growth when has been added to nutrition media but, the number of auxiliary buds decreased at concentration of 50% of silver nitrate. which proves that the concentrations 30 and 40% are extremely satiable for pepper vegetative growth in general whereas, the treatment of 50% recorded 3.6 explants. Moreover the lowest recorded results were obtained in treatment of control and 10%. This recorded results were in the same line with (Sharma *et al*., 2008) who found that the treatment of 30% silver nitrate obtained the best results and addition of silver nitrate has a positive effect on vegetative growth and flowering stage in pepper plant.

For number of leaves parameter the results proved that the addition of silver nitrate led to increase in number of leaves pepper plant variety of Maserati whereas, the treatment of 30% silver nitrate gave the best results and recorded a number of leaves reached to 12.4 leaves which was significantly higher than other used treatments. These obtained results proved the positive effect of silver nitrate on plant growth and explained the importance role for silver nitrate in pepper plant growth. Nevertheless these results proved also that the high levels of silver nitrate led to negative effect on number of leaves formation and plant growth in general which has been proved in several studies. Our obtained results were in the same line with (Fuentes *et al.,* 2000) (Christopher *et al*., 1996) who reported that addition of silver nitrate with concentration of 30 to 60 µM/L increased the plant growth of *(Coffeacanephora*) plant whereas, the high levels of silver nitrate led to negative effect on plant growth in general. Furthermore the researcher reported also that high levels act as an ethylene.

blocker and affect the amount of Abscisic acid inside the plant (Kong and Yeung, 1994). However several studies proved that present of ethylene inside the plant cell induces the plant to produce callus and also positively affect the formation of auxiliary buds which may useful in plant tissue culture studies (Giridhar *et al*., 2004 & Sharma *et al*., 2008 &Takasaki *et al*., 2004).

According to our obtained results we found that, the addition of silver nitrate with concentration of 30 and 40µM/L clearly increase the plant growth in general. Furthermore, the high concentrations of silver nitrate led to negative effect on plant growth whereas, the response to silver nitrate tend to be higher in variety of Maserati than Chile Ancho variety.

Table (3) Effect of silver nitrate (AgNo3) on callus formation, vegetative growth, Auxiliary buds and number of leaves after 90 days of growth (variety of Maserati)

Conc.AgNO3 µM/L	Callus formation (%)	Vegetative (%)	Auxiliary buds (%)	Number of leaves
0.0	**94** [a]	**15** [c]	**4.6** [bc]	**3.2** [c]
10	**75** [c]	**13** [d]	**2.2** [d]	**7.6** [b]
20	**80** [b]	**35** [ab]	**3.8** [bc]	**7.8** [b]
30	**90** [a]	**49** [a]	**5.6** [a]	**12.4** [a]
40	**75** [c]	**43** [a]	**5.3** [ab]	**9.5** [ab]
50	**60** [d]	**26** [bc]	**3.6** [cd]	**7.3** [b]

* Vertically similar letters, there are no significant differences between them using Duncan's multiple range test at the 5% level.

Effect of copper sulfate on callus induction and plant growth for Chile Ancho and Maserati varieties.

The results of this study (table 5 and 6) showed that the addition of copper sulfate improves the plant growth for both variety. The results proved that for callus formation parameter it has been found that there was significant differences among the used treatments whereas, the control treatment gave the highest quantity of callus this treatment recorded a percentage of 80 and 94% in Chile Ancho and Maserati variety respectively. However this recorded results explained that there was

no response to callus formation from both varieties. Furthermore for copper sulfate treatments the results showed that the treatments of 3 and 4µM/L copper sulfate obtained the best results whereas, those treatments recorded 50% of callus formation in Chile Ancho variety and 65% in Maserati variety.

Table (4): effect of (CuSO4) on callus formation, vegetative growth, Auxiliary buds and number of leaves after 90 days of growth (variety of Chile Ancho).

Conc.$CuSO_4$ µM/L	Callus formation (%)	Vegetative (%)	Auxiliary buds (%)	Number of leaves
0.1	80 ^{a*}	35^{a}	2.6b^{c}	3.4 ^{d}
0.5	25 ^{c}	10^{c}	2.2 ^{c}	4.8 ^{c}
1.0	25 ^{c}	15 ^{bc}	2.4 ^{c}	4.3 ^{c}
2.0	45 ^{b}	25 ^{ab}	3.5 ^{ab}	6.2 ^{b}
3.0	50 ^{b}	30 ^{a}	4.5 a	7.2 ^{a}
4.0	50 ^{b}	25 ^{ab}	4.6 ^{a}	6.8 ^{ab}

* Vertically similar letters, there are no significant differences between them using Duncan's multiple range test at the 5% level.

Addition of copper sulfate improves the plant growth in general but the exact concentration of copper sulfate should be studied carefully. Thereby several studies have been applied and studied the effect of copper sulfate on plant growth.

The results of this study showed that the addition of copper sulfate improved that plant growth while, treatment of 3µM/L copper sulfate obtained the best results whereas, recorded a percentage of 30% in Chile Ancho variety and 45% in Misraty variety. Moreover the auxiliary buds formation increased by the addition of copper sulfate whereas, the highest number of auxiliary buds was recorded in treatment of 4µM/L copper sulfate in both varieties whereas, the results recorded an average of 4.6 in Chile Ancho variety and 4.5 in Maserati variety, this obtained results proved the positive effect of copper sulfate on plant growth when added in satiable concentration. On the other hand the same has been found in number of leaves parameter while the number of leaves increased by the addition of copper sulfate and the treatment of 3µM/L of copper sulfate gave the highest number of leaves followed by the treatment of 4µM/L whereas, those treatments gave an average of number of leaves was significantly higher than the other treatments and those treatments recorded an average of 7.2 and 7.5 for each plant. Our results were in agreement with (Ghaemi *et al.*, 1994) who proved that the addition of copper sulfate improve the plant growth. Furthermore this results were in same line with Kothari and Joshi (Joshi and Kothari, 2007) who fund that the addition of copper sulfate increase the plant growth of some pepper variety. In addition our results were in full agree with (Dahleen, 1995) who fund that the addition of copper sulfate improve the callus formation of some barely varieties.

Table (5): effect of (CuSO4) on callus formation, vegetative growth, Auxiliary buds and number of leaves after 90days of growth (variety of Maserati)

Conc.$CuSO_4$ µM/L	Callus formation (%)	Vegetative (%)	Auxiliary buds (%)	Number of leaves
0.1	94 ^{a*}	15 ^{c}	4.6 ^{a}	3.2^{d}
0.5	30 ^{c}	15 ^{c}	2.4 ^{b}	5.1 ^{bc}
1.0	30 ^{c}	20^{b}	2.7^{b}	4.4 ^{cd}
2.0	50 ^{b}	30^{ab}	3.2 ^{ab}	6.6 ^{b}
3.0	65^{b}	45^{a}	3.8 ^{a}	7.5 ^{a}
4.0	65 ^{b}	25 ^{b}	4.5 ^{a}	7.2 ^{ab}

* Vertically similar letters, there are no significant differences between them using Duncan's multiple range test at the 5% level.

Conflict of Interest: The authors declare that there are no conflicts of interest.

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