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In vitro culture protocol, micropropagation, acclimatization and chemical constituents of *Spathiphyllum cannifolium* plant under copper concentration effect

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Abstract : The present work was carried out at tissue culture laboratory of Ornamental Plants and Woody trees Department to establish propagation protocol affecting by BA and NAA at different concentrations and study the effect of different copper concentrations in the culture medium on the *in vitro*and *in vivo* shooting behavioras well as chemical constituents of *Spathiphyllum connifolium* plants. For culture establishment, the results showed that supplementation of MS culture medium by 0.5 mg /l of BA gave the maximum shootlets numbers / explant. BA at 0.5mg/l + NAA 0.1 mg/l gave the longest shootlets. Copper was added to the medium at concentrations 0.0, 2.5, 5.0, 10.0 and 20.0 ppm. The highest number of shootlets, longest shootlets and the heaviest shootlets and fresh and dry weights of roots were obtained by the copper treatments at the low concentrations. After adaptation, copper at 2.5 ppm gave the highest values of shoot number, shoot length, leaves number, root number, root length, shoot and root fresh weight and plant dry weight. Chlorophyll (a), (b) and carotenoids as well as copper content were decreased by increasing copper concentration. All copper concentrations increased carbohydrates% but decreased nitrogen and zinc content, copper at 5.0 ppm increased P and K content.
Key words:*Spathiphyllum cannifolium,* plants, *in vitro,in vivo,* BA, NAA, copper.

Introduction

Spathiphyllum connifolium belongs to the family Araceae, it is a significant tropical ornamental plant that alluring foliage, its flowers consist of a white spathe and spandix. The plant is perennial and evergreen herbaccous with large leaves that reach 12 to 65cm long and 3 to 25 cm wide. The plant can survive with little light and water[1]. In the markets spathiphyllum is mostly sold without it flowers. A lot of the plant's types are commonly planted as indoor house plants as the plant is capable of survivingin shade with little sunlight and can only be watered once a week. The clean air study of Nasa revealed that spathiphyllum plant cleans the air of specific environmental contaminants including benzene and formaldehyde[2]. A few *in vitro* studies on micropropagation of this plant are present.

The procedures of consecutive micropropagation of many plants can be successful when influenced by various factors from which the most important once are growth regulators, physical conditions and growing media. Benzyladenine (BA) and Naphthalene acetic acid (NAA)'s effect at different concentration on the rate of the multiplication of shoot and root were recorded by [3] on *Ruscushypoglossum* L and [4] on *Deutzia scabra*. Techniques of *in vitro* paved the way to a lot of researches done on growth which enabled the researchers to

distinguish single parameters measuring their effect on growth. Such investigations focus on cytokinins,auxins and copper [5,6].

Cytokines and auxins are components of growth stimulants. Various plants have either obtained growth increment or growth inhibition after cytokines or auxins application [7,8].

Micro nutrients such as copper are responsible for the plant growth process and plant development [9,10,11]). Copper is a redox – active transition metal that has various roles such as being a cofactor for a lot of enzymes it plays a role in the photosynthesis and respiration processes. Additionally, it takes part in signaling hormones and transcription, protein trafficking machinery, lignin formation inside cell walls and in oxidative stress responses [9,11]. Also, copper is considered an important component or activator of a lot of enzymes that take part in the transport of electrons. Copper has a significant function in the biosynthesis of cell wall, transduction of signal and lignification of cell wall [12].

A number of authors noted in their research that higher concentrations of copper have a positive effect on*in vitro* cultures of different plant species. High copper concentration has an enhancing effect on morphogenetic responses of various species as revealed by Garcia –[13] on melon (*Cucumis melo*L.), [14] on *Withania somniferaus* L. (Indian ginseng) and [15] on *Daucus carrota* L. (Carrot). A member of reports have also revealed negative effect of higher copper concentration on *in vitro* regeneration, e.g. [16] on *Nicotiano Tabacum* L. and [15] on *Daucus Carrota* L. Copper can also have an effect on the accumulation and uptake of several essential metals [17]. Some micronutrients (Cu, Fe and Zn) play vital roles in plant metabolism.

The aim of this paper was to establish a micro propagation protocol of *Spathiphyllum cannifolium* and study the effects of elevated levels of copper (0.0, 2.5, 5.0, 10.0 and 20.0 ppm) in the culture medium on micro propagation behavior during developmental stages.

Materials and Methods

This work was carried out at tissue culture Laboratory of Ornamental Plants and Woody Trees Department, National Research Centre (NRC), Egypt during years 2014 and 2015 to establish *in vitro* micropropagation protocol and investigate the effect of cupper concentration in the culture medium on *in vitro* and *in vivo* growth behavior as well as chemical composition of *Spathiphyllum cannifolium* plant.

Surface sterilization:

Explants were gathered from mature plants, kept in polyethylene bags and transferred directly to laboratory, where they were immediately washed under running tap water for one hour.Surface sterilization were in 70% ethanol for 30 secthen immersed in 15% sodium hypochlorite NaOH (Clorox+0.01% Tween 20) for 7 minutes and then rinsed with sterile water three times. The explants were then sterilized in 0.1% HgCl2 solution for 5 minutes, rinsed five times in sterile water.

Establishment of *in vitro* cultures

For establishment stage, each three explants were cultured after sterilization process on solid MS media [18] free of hormones. The pH of the medium was adjusted to 5.7-5.8 to adding agar (8g/l) and sucrose (25g/l). Media was autoclaved at121ºC and 1.5 kg. Cm^2 for 20 minutes. Cultured explants were kept in growth chamber illumination of 16 hr photoperiod, 2000 Lux at 25±2ºC for 6 weeks to promote shootlet development. At the end of six weeks intervals, in order to obtain sufficient number of shoots,the shoots were sub-cultured on MS medium supplemented with various concentrations (0.0, 0.5, 1.0 and 2.0 ppm) of BA (6-Benzylaminopurine)individually or in combination with NAA at (0.0,0.1, 0.2 and 0.3 ppm), the following parameters were recorded: number of shootlets /explant, number of leaves/shootlet and length of shootlets.

Copper treatments

After two months, newly formed shoots about 20 mm long were separated, individually transferred (3 shoots per vessel, and 30 per treatment) to MS multiplication medium (Fig. 1) supplemented with Copper (as $CuSO_4 \times 5H_2O$) was added in different concentrations(0.0, 2.5, 5.0, 10.0 and 20.0 ppm). The number and length of shoots, leaves number, and the fresh, dry weights of shoots and roots were analyzed after eight weeks of growth. This experiment was repeated twice.

Fig. (1): Developmental stages of *Spathiphyllum canifolium* in MS medium supplemented with different concentrations of copper.

Acclimatization of micropropagated plants

Shoot number/ plant, shoot length(cm), leaves number/ plant, root number /plant, root length (cm), shoot and root fresh weight(g), and plant dry weight(g) were recorded (Fig. 2).

Fig. (2): Acclimatized plants of *Spathiphyllum canifolium* under various copper concentrations effect.

Chemical analysis:

Determination of pigments content (mg per 100 g F.W) of chlorophyll a, b and carotenoids was carried out according to the method described by[19].

Total carbohydrate percentagein the dry samples after acclimatization was determined according to [20].

Mineral content

Nitrogen content was determined by modified micro-Kjeldahl method as described by[21]. Phosphorus content was estimated (after wet ashing) using ammonium molybdate method according to[22].Potassium was determined by using flame photometer according to[23].Calcium, zinc, iron and copper were determined using atomic absorption spectrophotometer according to[24].

Statistical analysis:

Data were statistically analyzed according to Duncan's multiple range tests at 5% level of probabilityas the method described by[25].

Results and Discussion

Culture establishment

In this stage, the effect of culture media supplemented with BA at various concentrations (0.0, 0.5, 1.0 and 2.0 ppm) individual or in combination with NAA at (0.0,0.1, 0.2 and 0.3 ppm) on the *in vitro* shootingbehavior of *Spathiphyllum Cannifolium*was investigated (Table 1). Results revealed that, supplementation of MS culture medium by 0.5 mg /l of BA gave the maximum shootlest numbers / explant (9.8) followed by 1.0 mg/l of BA (4.3) and then BA at 0.5 + NAA 0.1 mg/l (3.67) compared with the control plant (1.67). In this respect, BA at 0.5mg/l + NAA 0.1 mg/l gave the longest shootlets length (61.67mm) followed by BA at 0.5mg/L. (55.0mm) and then BA at 1.0 + NAA at 0.2 mg/l (50.0mm) comparing with untreated plants (22.5mm). In addition, the highest leaves number / shootlet were obtained by BA at 2.0mg/l (33.67) followed by BA at 1.0 mg/ l(24.33) or BA at 2.0+ NAA at 0.3mg/l(14.67) comparing with control plants (5.33).

Table 1: Effect of BA and NAA on *in vitro* shooting behavior of *Spathiphyllum Cannifolium*

Treatments	Shootlets number/explant	Shootlets length(mm)	Leaves number
Control	1.67	22.50	5.33
BA at 0.5 ppm	9.80	55.00	14.67
BA at 1.0 ppm	4.30	31.67	24.33
BA at 2.0 ppm	3.50	37.00	33.67
BAat 0.5+NAA0.1ppm	3.67	61.67	10.33
BAat 1.0+NAA0.2ppm	5.20	50.00	7.00
BAat 2.0+NAA0.3ppm	1.66	22.50	11.67
LSD at 0.05	1.63	8.84	7.40

We could explain these results through the fact that cytokines have important physiological effects explaining theresults given previouslysince they have beenshown to stimulate cell divisionas well as the elongation of cell which stats the synthesis of RNA and induces the synthesis of protein and activity of enzymes [26,27]. Higher cytokinins levels were used very effectively as method to decrease shoot and leaf growth and enhance the formation of meristematic clusters [28].

Effect of different copper concentrations on micropropagation behavior

The data obtained (Table 2) on *in vitro* culture indicated that mean values of morphological traits were increased with increasing Cu concentrations from 2.5 to 10ppm in the culture medium. The highest shootlets number/ explant (2.22) was noted with 2.5 ppm treatment as well as the longest shootlets (40.0mm) were

noticed with 10 ppm Cu treatment versus the control (30.22mm). The leaves number/ shootlet showed a decreasing trend following the increasing Cu concentration in the culture medium. The heaviest shootlets fresh weights (0.423g) were recorded in the treatment 2.5ppm Cu. This means that values significantly differ from the control treatment. The same results were obtained with 2.5 ppm Cu on shootlets dry weight. The heaviest shootlets dry weight (0.423g) was resulted from 2.5 ppm Cu treatments. Fresh and dry weights of roots were increased by all Cu concentrations treatments. They showed an increasing trend following the decreasing Cu concentration in the culture medium. 20ppm of Cu treatment led to lower number of shootlets/explant, shootlet length, leaves number and fresh and dry weight of shootlets than control. The lightest fresh and dry weights of roots were obtained by the control. According to the obvious results obtained, the rate of growth *in vitro* can be increased in the media containing lower concentrations of copper and gave the high quality of growth characters [29]. Micronutrients in plant cells, especially copper is important in the process of respiration where copper is the functional parts of some oxidative enzymes contained in plant tissues [30].

Table 2: Effect of various copper concentrations on *in vitro* shooltlet multiplication of *Spathiphyllum Cannifolium*

Treatments	Shootlets number/ explant	Shootlets length(mm)	Leaves number	Shoot fresh weight(g)	Shootdry weight (g)	Root fresh weight(g)	Root dry weight(g)
Control	1.22	30.22	6.50	0.239	0.033	0.129	0.014
Cu at 2.5 ppm	2.22	38.17	6.06	0.423	0.061	0.553	0.039
Cu at 5.0 ppm	2.14	37.22	5.33	0.301	0.040	0.310	0.031
Cu at 10.0 ppm	1.67	40.0	5.17	0.265	0.036	0.403	0.030
Cu at 20.0 ppm	1.00	20.67	3.33	0.219	0.022	0.175	0.018
LSD at 0.05	0.65	10.49	2.07	0.044	0.016	0.044	0.009

Table 3: Effect of various copper concentrations on growth behavior of acclimatized *Spathiphyllum cannifolium* plantlets

Treatments	Shoot number /plant	Shootlength (mm)	Leaves number /plant	Root number /plant	Rootlength (cm)	Shoot fresh weight(g)	Root fresh weight(g)	Plant dry weight(g)
Control	1.00	7.33	7.33	6.33	7.50	0.70	0.216	0.108
Cu at 2.5 ppm	2.00	7.60	8.00	11.33	9.00	0.63	0.283	0.178
Cu at 5.0 ppm	1.67	6.93	5.83	6.60	8.67	0.47	0.370	0.037
Cu at 10.0 ppm	1.00	3.76	6.50	5.67	6.00	0.33	0.133	0.075
Cu at 20.0 ppm	1.00	3.00	5.83	3.00	6.00	0.25	0.123	0.048
LSD at 0.05	N.S	1.58	1.44	1.77	2.36	0.113	0.048	0.023

Effect of different copper concentrations on growth behaviorofadapted plantlets

Results in Table (3) showed that shoot number of *Spathiphyllum cannifolium* plantlets were increased with 2.5 and 5.0 ppm Cu treatments as compared with control and other treatments. The highest value of shoot number (2.0) attained from 2.5ppm Cu followed by plant treated with 5.0ppm Cu (1.67). Plantlets produced from the lowest concentration of cupper (2.5 ppm) gave the highest values of shoot length, leaves number and roots number (7.6, 8.0 and 11.33), respectively. The results also indicated that Cu at 2.5ppm gave the longest roots (9.0cm) followed by Cu at 5.0 ppm (8.67cm). The maximum value of shoot fresh weight (0.7g) was obtained by 2.5 mg/l Cu treatment but the maximum value of root of fresh weight resulted by 5.0 ppm of Cu treatment. Plant dry weight was increased by Cu at 2.5 ppm compared with control. The lowest values of the obvious growth characters were obtained by the high concentration of Cu (20ppm) except shoot number, leaves number and plant dry weight. In general, the low concentration of Cu (2.5ppm) was most effective on growth parameters of acclimatized plantlets of*Spathiphyllum cannifolium,* and the growth values were decreased by increasing Cu concentrations.

These results may be due to that, the copper participates in numerous physiological processes and is as essential cofactor for many metalloproteinase, however, problems arise when excess copper is present in cell. Excess copper inhibits plant growth and impairs important cellular processes [31]. Excess copper can cause

disorders in plant growth and development by adversely affecting important physiological process in plants. Thus, at high concentrations, Cu can become extremely toxic causing symptoms such as chlorosis and necrosis, stunting, leaf discoloration and inhibition of root growth [32,33,34].

Photosynthetic pigments

Regarding the effect of copper applications on pigment contents, the obtained results in Table (4) cleared that raising the levels of copper used decreased chlorophylls a, b and carotenoids shootlets contents, i.e., applying the plants with copper at 20mg/l resulted in the lowest values whereas, the highest values of pigments content were obtained by control plants. (13.98, 11.35 and 22.89 mg/100g F.W., respectively). Generally, pigment contents were decreased by increasing copper concentrations. This may be attributed to the decrease of photochemical activity caused by Cu is accompanied *in vivo* by an alteration of the structure and composition of the thylakoid membranes which can influence the conformation and function of the photosystems [35,36,37]. These results are in a harmony with[31].

Table 4: Effect of various copper concentrations on photosynthetic pigments content (mg/100g F.W.) and carbohdrates% content of *Spathiphyllum cannifolium* shootlets

Treatmeats	Chlorophyl(a)	Chlorophyl(b)	Carotenoids	Carbohydrates%
Control	29.40	15.53	56.18	24.37
Cu at 2.5 ppm	23.42	14.53	55.58	26.77
Cu at 5.0 ppm	21.08	13.92	97.27	29.30
Cu at 10.0 ppm	16.75	12.16	39.20	34.50
Cu at 20.0 ppm	13.98	11.35	22.89	29.17
LSD at 0.05	1.61	0.501	0.302	0.503

Carbohydrates content%

As shown in Table (4), the percentage of carbohydrates plant content was increased with copper treatments compared with the control. The maximum value of carbohydrates percentage (34.50%) was obtained when culture medium was supplemented with Cu at 10.0 ppm. However, the lowest percentage of carbohydrates resulted from control treatment. Like in many other plants, the transport differential Cu may antagonistically affect the uptake and accumulation of other essential inorganic nutrients [17].

Elements content%

Data on macroelements percentages of *Spathiphyllum connifolium* plants, as affected by different copper concentrations, are presented in Table (5). All copper concentrations decreased nitrogen percentage compared with the control. The average N% ranged from 1.07 to 1.67%. The untreated plants were the highest one (1.67%) whereas the lowest value (1.07%) was recorded on plants treated with Cu at 20.0ppm.

The results in the same table indicated that the maximum content of P% (0.28%) was recorded on plant treated with Cu at 5.0 ppm but the lowest value (0.13%) resulted from plants treated with Cu at 20.0 ppm compared with the control. In regards to the effect of different concentrations of copper on potassium % of plants, the average percentage of K% ranged from 0.43 to 0.87%. Plants treated with 5.0ppm Cu gave the highest value of K% (0.87%), whereas, Cu at 20.0ppm gave the lowest percentage of K% in plants (0.43%) compared with the other concentrations.

Table 5: Effect of various copper concentrations on elements content of acclimatized *Spathiphyllum cannifolium* plants

Treatments	Nitrogen	Phosphorus	Potassium	Calcium	Zinc	Iron	Copper
Control	1.67	0.196	0.80	0.20	0.457	1.128	0.085
Cu at 2.5 ppm	1.36	0.176	0.80	0.34	0.018	1.175	0.081
Cu at 5.0 ppm	1.51	0.280	0.87	0.25	0.087	1.997	0.128
Cu at 10.0 ppm	1.31	0.190	0.64	0.16	0.129	0.408	0.585
Cu at 20.0 ppm	1.07	0.130	0.43	0.20	0.078	1.043	0.725
LSD at 0.05	0.20	0.048	0.11	0.04	0.002	0.004	0.002

Concerning the effect of copper concentrations on calcium %, the maximum percentage of Ca (0.34%) was obtained from plants treated with Cu at 2.5ppm followed by Cu at 5.0ppm (0.25%) compared with the other treatments. On the other hand, using Cu at10.0 ppm resulted the lowest K%(0.16%).Copper at low concentrations had a positive effect on Ca accumulation in plants. Calcium is a vital macronutrient for plants, for example it has an important function in intercellular signaling, stomatal regulation and cell wall stabilization [38].

Iron contents were increased by treated plants with Cu at 2.5 or 5.0 mg/l treatments compared with the control plants .The highest value of Fe content (1.997 ppm) was obtained by Cu at 5.0 ppm treatment. However, the lowest value of Fe content resulted from Cu at 10.0 ppm treatment compared with the other treatments.

Zinc content in plants tended to decrease with all copper concentrations in the culture medium .The untreated plants gave the maximum value of Zn (0.457ppm)whereas, the lowest one (0.078ppm) was obtained with Cu at 20.0 ppm compared with the other treatments. Zinc is a vital minor element, which has many functional, regulatory and structural roles in a lot of enzymes. This element is also significant for the production of auxins, metabolism of carbohydrates and proteins, protection of cells against oxidative stress, photosynthetic reaction and the membrane structure and functions maintenance [39].The toxicity of zinc stimulates chlorosis in plants, either by competing with the uptake of other elements or due to reduction in the synthesis of chlorophyll because of deficiency [40,41]. Increasing copper concentrations in the culture medium led to a marked increase in copper content. The maximum value in copper content (0.725 ppm) was observed from Cu at 20.0 mg/l treatment. Hence, we recommend that, using culture medium containing copper at low concentrations markedly increased both *in vitro*and *in vivo* vegetative growth as well as nutrients content of *Spathiphyllum connifolium* plants.

Conclusion

Using culture medium containing copper at low concentrations markedly increased vegetative growth of micropropagated and adapted plants as well as nutrients content of *Spathiphyllum connifolium.*

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