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MORPHOLOGICAL AND CHEMICAL STUDIES ON THE EFFECT OF THE GROWTH RETARDANT 'CYCOCEL' ON MICROPROPAGATION OF *ANANAS COMOSUS* CV. QUEEN

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Abstract

Pineapple is a greenhouse promising crop in Egypt. It attracts attention of investors and exporters. Pineapple *in vitro* culture presents practical method for providing large number of plants for cultivation or export. This investigation aimed to study the effect of cycocel (CCC) on multiplication and rooting of pineapple plants grown *in vitro* with various concentrations (0.75, 1.25, 2.5 and 5 mg/l). Results showed that, all CCC concentrations affected shoot multiplication of pineapple *in vitro* culture as shoot number, length and leaf number, significantly. It was clear that, average number of shoots was increased gradually by increasing cycocel concentrations to 5.0 mg/l while shoot length showed the reverse. When the lowest concentration (0.75 mg/l) was added to rooting medium it enhanced root number, root length and plant thickness. Biochemical analysis showed that pigments, total phenols and tannins were increased by raising concentrations of cycocel in the medium, as their values reached the highest at 5.0 mg/l. Similarly, adding cycocel to the medium raised the activity of catalase, superoxid dismutase and glutathione peroxidase enzymes in pineapple shoots compared with the control.

Key words: antioxidant enzymes, growth retardant, cycocel, in vitro, pineapple.

Introduction

Pineapple (*Ananas comosus* L.) is an important fruit crop cultivated in tropical and subtropical regions of the world. Pineapple belongs to family Bromelaceae, is a perennial monocot having a terminal inflorescence and fruit. The fruit is a good source of vitamins A, B and C, calcium, phosphorus and iron. The dry weight of fruit contains 75-83% sugars and 7-9% citric acid (Flath, 1980). Pineapple is a rather promising new crop in Egypt to be grown in plastic greenhouse especially in the newly reclaimed lands.

Growth retardants (absisic acid, ancymidol, paclobutrazol, chlormequat, etc.) are usually used to suppress growth of plants either *in vitro* or *ex vitro*. It can be used for slow growth conservation (Hassan *et al*., 2016). Conversely, it can be used for enhancing multiplication, embryogenesis, germination, rooting of some *in vitro* plants like date palm (Ibrahim *et al*., 2011).

Chlormequat (2-Chloro-N, N, N-trimethylethana minium chloride) is an organic compound that is used as a plant growth regulator. It is typically presented as the chloride salt; chlormequat chloride or chlorocholine chloride (commercially cycocel; CCC). It is considered as the most important inhibitor of gibberellins biosynthesis (Rademacher and Brahm, 2010). It might be used for hardening, increasing tolerance of the plant (Thakur *et al*., 2016).

This investigation was carried out to study the effect of cycocel (CCC) on multiplication and rooting stages of pineapple *in vitro* cultures.

Materials and Methods

This study was carried out in Biotechnology and

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Plant material and explants preparation

Shoot-tips of pineapple (*Ananas comosus*) cultivar "Queen" were taken from crown of mature fruits of about 20-25 cm in length. The older leaves were carefully removed. Shoot tip containing the apical meristem and 2-4 leaf primordials were washed under tap water for half an hour. The isolated shoot tip explants were soaked under aseptic condition in 15 % Clorox (5.25 % sodium hypochlorite) for 20 minutes with one drop of Tween 20. The explants were then rinsed several times using sterilized distilled water.

Establishment of explants

Sterilized shoot-tips were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg/l BA + 0.1 mg/l NAA + 0.4 mg/l thiamine-HCl + 30 g/l sucrose + 6 g/l agar (Bader El-Deen, 2003). After eight weeks all survived explants were transferred and recultured on the same medium. The culture medium was distributed in culture jars (325 ml), where each jar contained 50 ml of the prepared medium. The culture jars were immediately capped with polypropylene closures and autoclaved at 121°C and 15 Ibs /ins^2 for 20 min. After three months of cultures, *in vitro* generated shoots were used in this study.

Cycocel treatments

A primary experiment had been established to determine the suitable concentration of cycocel (CCC). The concentrations of 5, 10, 20 and 40 mg/l were investigated. Unpublished results showed that 20 and 40 mg/l had a lethal effect on *in vitro* shoots at multiplication and rooting stages Fig. 1. A new experiment had been established with lower concentrations as 0.75, 1.25, 2.5 and 5 mg/l at multiplication and rooting stages. Multiplication medium was supplemented with previous components, while rooting medium was supplemented with 1.0 mg/l IBA. (Bader El-Deen, 2003).

Incubation Conditions

Pineapple shoots were incubated at temperature of 25 ± 2°C. Light was provided by white fluorescent lamps, giving the intensity of about 3000 Lux for 16 hours daily.

Data Recorded

Average number of shoots, average number of leaves and shoots length (cm) were measured in multiplication stage. In addition, rooting percentage, root number, root length, average plantlets length and plantlets thickness were determined in rooting stage.

Adaptation of Plantlets

Explants were transplanted after rooting to greenhouse and washed with tap water for three times to remove all traces of agar then soaked in a systemic fungicide at 1g/l then cultured in black plastic pots containing a mixture of peat moss and sand 1:1 (v/v). Plantlets were covered with transparent plastic bags to maintain humidity up to 80-90%. Plastic bags were removed gradually after 7-10 days and fully removed after 14 days. Plantlets were irrigated with tap water regularly when needed.

Extraction and determination of pigments and secondary metabolites

Pineapple *in vitro* shoots were taken at the end of the multiplication experiment in order to determine photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoides) according to Yang *et al*., (1998). In addition, total flavonoids (Zhishen *et al*., 1999), phenols (Singleton *et al*., 1965) and tannins (Polshettiwar *et al*., 2007) were measured.

Extraction and determination of antioxidant enzymes activities

Fresh plant material (0.1g) was homogenized in 5 ml of ice-cold buffer phosphate (pH 7.4). The homogenate was centrifuged at 10000 rpm for 30 min and supernatant was collected. The resulting supernatant was used for determination of enzyme activities. The superoxide dismutase, glutathione peroxidase and catalase activities in plant homogenate were measured spectrophotometrically by using Stanbio enzymatic SOD kit.

Statistical Analysis

Data were analyzed as one-way completely randomized factorial design and means separated by Steel and Torrie (1980) at a 5% level of significance.

Results

Data in table 1 revealed that all used CCC concentrations affected shoot multiplication of pineapple in vitro culture, significantly. It is clear that, average number of shoots was increased gradually by increasing cycocel concentrations to 5.0 mg/l as it showed the highest significant value (13.00). In addition, leaf number was increased by increasing cycocel concentrations to 2.5 mg/l to reach (5.48). Meanwhile, shoot length was negatively affected by all concentrations of cycocel compared with the control.

It is clear from data in table 2 that, when the lowest

concentration (0.75 mg/l) was added to rooting medium it enhanced root number (4.33), root length (7.08) and plant thickness (1.55). Furthermore, that concentration remained rooting percentage values without any significant differences with the control (100%). Meanwhile, plant length was inhibited by all concentrations Fig. 2.

Table 1: Effect of cycocel concentration on shoot multiplication of *Ananas comosus*.

Cycocel (mg/l)	Shoot number	Shoot length (cm)	Leaf number
0.00	10.33d	1.02a	5.27b
0.75	11.33c	0.81b	5.43ab
1.25	11.25c	0.80b	5.45a
2.50	11.58b	0.77b	5.48a
5.00	13.00a	0.48c	4.00c

Means with different letters within each column were significantly different at 5% level.

Table 2: Effect of cycocel concentration on *in vitro* rooting of *Ananas comosus*.

Cycocel (mg/l)	Rooting %	Root number	Root length (cm)	Plant length (cm)	Plant hickness (cm)
0.00	100	4.00b	6.54b	4.89a	1.02b
0.75	100	4.33a	7.08a	4.61b	1.55a
1.25	100	3.31c	5.69c	3.89c	0.93bc
2.50	88.89	3.00d	5.11d	3.17d	0.87c
5.00	88.89	2.91d	3.29e	3.11e	0.71d

Means with different letters within each column were significantly different at 5% level.

Data in table 3 indicated that Chlorophyll a, b, a+b and carotinoide of pineapple shoots were increased by raising the concentration of CCC in the medium as their values reached the highest at 5.0mg/l.

Similarly, total phenols and tannins were increased by raising concentrations of cycocel in the medium as their values reached the highest at 5.0 mg/l (32.09 and 16.17, respectively). Meanwhile, total flavonoids had inverse response to the presence of cycocel in the medium (Table 4).

It is clear from data in table 5 that antioxidant enzymes activity was affected with the presence of CCC in the medium. Raising cycocel concentration to 2.5 mg/L gave the highest activity value of Catalase enzyme compared with the control and without any significant differences with other cycocel concentrations investigated. Similar results were obtained with Superoxiddismutase and Glutathione peroxidase as they showed the highest activity with higher CCC concentrations used compared with the control.

Table 3: Effect of cycocel concentration on chlorophyll a, b, a+b and carotinoide of pineapple shoots.

Cycocel (mg/l)	Chlorophyll a (µg/g)	Chlorophyll b (µg/g)	Chlorophyll a+b (µg/g)	Carotinoide (µg/g)
0.00	15.74d	6.61e	22.34d	4.12c
0.75	20.94c	10.72c	31.66c	4.37c
1.25	22.08bc	10.49d	32.58c	5.30b
2.50	23.50b	11.16b	34.66b	5.92ab
5.00	26.66a	13.70a	40.36a	6.68a

Means with different letters within each column were significantly different at 5% level.

Table 4: Effect of cycocel concentration on total Phenolics, flavonoids and tannins of pineapple shoots.

Cycocel (mg/l)	Phenolics mg*/g Dw	Flavonoids mg**/g Dw	Tannins mg***/g Dw
0.00	26.61e	20.04a	8.10c
0.75	27.75d	19.78a	8.84c
1.25	30.08c	16.22b	9.42c
2.50	31.16b	15.04b	11.96b
5.00	32.09a	15.44b	16.17a

Means with different letters within each column were significantly different at 5% level. *as Gallic acid, **as Quercetin, ***as Tannic acid.

Table 5: Effect of cycocel concentration on antioxidant enzymes activity of pineapple shoots.

Cycocel (mg/l)	Catalase (U/g)	Superoxiddismutase (U/g)	Glutathione peroxidase (U/g)
0.00	3.65b	24.57c	27.86b
0.75	4.36ab	30.83b	27.24b
1.25	4.27ab	31.98ab	27.81b
2.50	5.08a	32.77ab	31.08a
5.00	4.39ab	33.65a	31.34a

Means with different letters within each column were significantly different at 5% level.

Discussion

It is obvious from this investigation that in vitro pineapple shoots number was increased gradually by increasing cycocel concentrations used in this investigation. Similarly, the presence of growth retardant (paclobutrazole) with cytokinin were found to enhance multiplication of Habanero pepper (*Capsicum chinense* Jacq.) as percentage of shoots formation and shoot number per explant (Bello-Bello *et al*., 2010). In addition, Chen *et al.,* (2005) indicated that meristematic clusters were induced from daylily 'Scape' explants in the presence of the growth retardant Paclobutrazol on semisolid agar medium.

Cycocel was found to be an inhibition factor for in

Fig. 1: Effect of high concentrations of cycocel on multiplication stage; notice the lethal effect on shoots (treatments from left to right: 5, 10, 20 and 40 mg/l, unpublished data).

Fig. 2: Effect of cycocel on rooting stage; notice its effect on length (treatments from left to right: 0.75, 1.25, 2.5 and 5 mg/l).

vitro shoot length of pineapple compared with the control. Similar inhibition in plant length was observed by Ray and Bhattacharya (2008). This inhibition is probably due to cycocel anti-gibberellin activity (Anon, 2003). Fletcher and Gilley (2000) assured that growth retardants act as primarily by inhibiting gibberellin biosynthesis and through secondary modulation of ABA, ethylene, cytokinin and polyamine metabolism.

In this investigation, root number, root length and plant thickness were increased with the addition of CCC but at the lowest concentration used, without any suppression in rooting percentage. Similar results were obtained with Ray and Bhattacharya (2008) as they indicated that cycocel could enhance root number and root length of *Eclipta alba* microshoots significantly. Out of 4 concentrations (0.63, 3.16, 6.33 and 12.66 µM), 6.33 µM of CCC was found most effective for inducing certain beneficial changes in rooting. It may be due to overcoming the inhibition of rooting caused by gibberellic acid to shoots (Sebastian and McComb, 1986). Likewise, ancymidol as a growth retardant act as an anti – gibberellin and Desjardins *et al*., (1987) thought that it might decrease the activity of invertase enzymes because the rooting of shoots obtained from asparagus single node culture was improved in the presence of 5.0 µM ancymidol and 7% sucrose.

Photosynthetic activity, expressed as chlorophyll a, chlorophyll b, chlorophyll a+b and carotenoids, was stimulated by CCC treatment in this investigation. Chlorophyll, contents in leaves of plants treated with CCC were higher than those of control plants. This observation was ascribed to the higher metabolite accumulation in CCC-treated plants compared with the untreated ones (El Sayed and Ebrahim, 2012). In addition, chlorophyll content of *Eclipta alba* leaves was increased by about 6 times than that in the control by using cycocel in the medium (Ray and Bhattacharya, 2008 and Thakur *et al*., 2006). Furthermore, carotenoides content of tobacco tissue grown in suspension culture was significantly affected by 2 mg/l I-naphthaleneacetic acid (NAA) and 500 mg/l CCC. CCC caused a 4-fold increase of carotenoid concentration in the tissue and a 2-fold increase of carotenoid accumulation per one cultural flask mainly due to the appearance of significant amounts of lycopene (Gamburg, 1978).

It worth to be mentioned that increasing chlorophyll synthesis, plant biomass, plant thickness, root number and inhibition of stem elongation significantly increased ex-vitro survival frequency and that made plants stouter than the non-treated plants (Ray and Bhattacharya, 2008 in *Eclipta alba* and Thakur *et al*., 2006 in Daylily).

In this investigation, raising cycocel concentration increased antioxidant enzymes activity. Similar results had been obtained with Pakar *et al*. (2016) as they indicated that CCC significantly affected antioxidant enzymes like peroxidase, catalase, and superoxide dismutase as it increased their activity in barley.

Conclusion

Cycocel could be the solution for enhancing *in vitro* regeneration of *Ananus sps*. as its presence increased multiplication rate, rooting and all properties needed for successful acclimatization.

Conflict of Interest

The authors declare that present study was performed in absence of any conflict of interest.

Authors' Contributions

All authors contributed extensively to the work presented in this article. R.A. Taha: designed and performed research, statistical analysis, data interpretation, wrote and revised the manuscript. E.A. Ibrahim, A.A. Gaafar: Performed chemical analyses and reviewed the manuscript. N.S. Zaied: helped in designing research and discussion. All authors read and approved the final manuscript.

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