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Research Article

PIONEERING *in vitro* STUDIES FOR CALLUS FORMATION OF *Colchicum chalcedonicum* Azn.

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Abstract: *Colchicum calcedonicum* Azn is one of the endemic species distributed in Turkey, where many endemic plant species occur. It has long-oval shaped corm under the soil, and usually 3-4 leaves on it. *In vitro* production of endemic species using callus culture has become promising study for conservation. The aim of this study is to generate an efficient callus protocol for *in vitro* production of *C. chalcedonicum.* To sterilize the explants, 0.25% (w/v) mercuric chloride ($HgCl_2$) was used for 20 min. In addition to mercuric chloride, surface sterilization was conducted by using 6.5% NaCl with Tween 80 for 30 min. We used 19 different mediums and the primary callus formation was obtained in Murashige & Skoog's basal medium (MS) supplemented with 2,4-D (2 mg L^{-1}), 2IP (0.5 mg L^{-1}), 3% sucrose and 0.05% active carbon. Our study demonstrated the active carbon usage was effective for the primary callus formation. This study is the first report for primary callus formation of *C. chalcedonicum.* However, our work is a pioneering study to improve callus formation protocol system for *in vitro* conservation of endemic species *C. chalcedonicum.*

Özet: *Colchicum calcedonicum* Azn, pek çok endemik bitki türünün görüldüğü Türkiye'de yayılış gösteren endemik türlerden biridir. Toprak altında uzun-oval şekilli soğanı ile genellikle 3-4 yapraklıdır. Kallus kültürü kullanarak endemik türlerin in vitro üretimi, bu türlerin korunmasında umut verici bir çalışma haline gelmiştir. Bu çalışmanın amacı, in vitro C. chalcedonicum üretimi için verimli kallus protokolünün oluşturulabilmesidir. Explantların sterilizasyonunda, 20 dk %0.25 (w/v) cıva klorür ($HgCl_2$) kullanılmıştır. Cıva klorüre ilaveten, yüzey sterilizasyonunda 30 dk boyunca Tween 80, %6,5 NaCl ile birlikte kullanılmıştır. Bu çalışmada, 19 farklı besiyeri kullanılmış olup primer kallus oluşumu 2,4-D (2 mg L-1), 2IP (0,5 mg L-1), %3 sükroz ve %0,05 aktif karbon içeren Murashige & Skoog bazal besiyerinde elde edilmiştir. Çalışmamız, aktif karbon kullanımının primer kallus oluşumunda etkili olduğunu göstermiştir. Bu çalışma, C. chalcedonicum'un primer kallus oluşumu için ilk rapordur. Bununla birlikte, çalışmamız endemik tür olan C. chalcedonicum'un in vitro korunması ve kallus oluşum protokolünün geliştirilmesinde öncü bir çalışmadır.

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Conservation

Introduction

The genus *Colchicum* L. within the family Colchicaceae have been known for more than 2000 years for their marked beneficial and poisonous effects (Brickell 1984). In Turkey, 50 *Colchicum* species were described of which 15 are endemic. Their limited distribution at a very high altitude and the restricted period of growth are the reasons for the low yield of members of the genus. *Colchicum* includes about 200 perennial flowering species growing from corms and their ovary flowers form underground. The important species of the genus, *C. luteum* Baker and *C. autumnale* L. contain 0.2-0.5g 100g^{-1} dry wt. of colchicine, which is an antimitotic agent by preventing accumulation of microtubules and inhibiting the cell division in the metaphase (The Wealth of India 1962, Kapadia *et al.* 1972, Dumontet & Sikic 1999, Combeau *et al.* 2000, Pirildar *et al.* 2010).

Colchicum chalcedonicum Azn., which is also known as Kadıköy (Chalcedon) crocus, is one of the endemic species of *Colchicaceae* in Turkey. *Colchicum chalcedonicum* was collected in Kadıköy in İstanbul by Aznavour in 1897 (Aznavour 1897). It has usually 4 leaves and long-oval shaped corms under the soil. Their chromosome number is 2n=50. The plant grows in rich red soils in dry stony and rocky places. They are also found on rare peaks and shrubs. *Colchicum*

chalcedonium flowers from August to September, being out of leaves and fruits from February to April. Additionally, the plants only live underground for a part of the year, thus *in vivo* and *in vitro* micro-propagation and *in vitro* culture of this species are difficult making the species to be known as calcitrant (Brickell 1984, Persson 1988, 1998, 1999a, 1999b, 1999c, 2000, 2001, 2007, Akan & Eker 2005).

Several studies reported on production of colchicine alkaloid by plant tissue cultures (Hayashi *et al.* 1988, Yoshida *et al.* 1988). Callus tissues were first induced from flowering shoots of *C. autumnale* L. by using MS (Murashige & Skoog 1962) containing 2,4-Dichlorophenoxyacetic acid (2,4-D), while colchicine from callus tissue was produced by MS with indole butyric acid (IBA) and kinetin (Hayashi *et al.* 1988). Daradkeh *et al*. (2012) used *C. hierosolymitanum* Feinbrun for callus production on MS supplemented with 0.45 µM 2, 4-dichlorophenoxyacetic acid under dark conditions. To induce colchicine production, callus was sub-cultured every 27 days on the same liquid media supplemented with 0.54 µM 1- naphthaleneacetic acid. The researchers observed that higher cell fresh weight was resulted with 9 µM 6-benzyladenine with 0.45 µM 2, 4-dichlorophenoxyacetic acid. Additionally, the highest colchicine alkaloid (0.090 mg g^{-1} DW) was obtained at 0.1 M sucrose after 4 weeks incubation (Daradkeh *et al.* 2012). Different parts of *C. chalcedonicum* and *C. micranthum* Boiss., which are endemic for Turkey, were also investigated for cytotoxic activities for future medical approaches. Daradkeh *et al.* (2012) managed to isolate colchicine, colchifoline, 2-demethylcolchicine, demecolcine, 4-hydroxycolchicine and *N-deacetyl-N*-formylcolchicine which showed high cytotoxicity. The main alkaloids of these two *Colchicum* species were found as colchicine and colchifoline. According to the results of this study, the greatest diversity in tropolone alkaloids were found in the seed of *C. chalcedonicum* (Gulsoy-Toplan *et al.* 2018). Despite its importance, no systematic attempt has been performed for mass propagation of *C. chalcedonicum*. The restricted distribution of this endemic plant has endangered its survival. Therefore, tissue culture approaches are required to get rapid propagation as *in vitro* protocol for micro-propagation, but there is no a practical protocol available for *in vitro* mass propagation of *C. chalcedonicum*. In the present study, we report the first and efficient protocol for callus generation of *C. chalcedonicum* using corms.

Materials and Methods

Field Studies and the Plant Material

Colchicum chalcedonicum corms used in this study were kindly provided by Erdal Uzen from Kadıköy. Field studies were performed from April 2017 to November 2017 at Çamlıca Hill (Ferah neighbourhood) (Fig. 1). The specimen was obtained from seed, leave, and corm during seeding, blooming and fruit time of *C. chalcedonicum* (Fig. 2). The specimens are deposited in the Istanbul University Alfred Heilbronn Botanical Garden.

Corms were long-ovaloid like egg-shaped, and corm shells mostly with several layers, the outer coriaceous and dark brown, the inner thin and reddish-brown. Leaves were 3-4, hysteranthous, patent, and oblong-lanceolate, light or dark-green hereinbefore mentioned by Küçüker (1984).

Explant Preparation

The fresh corms, leaves and corm shells were used as explants. The explants were excised aseptically with sterile scissors and washed with running tap water for 30 minutes, washed with dH_2O for three times for 5 min. The explants were then sterilized with 0.25% (w/v) mercuric chloride ($HgCl_2$) solution for 20 min. followed by surface sterilization with 6.5% NaCl with Tween 80 for 30 min. Then, the explants were rinsed thoroughly with sterile dH_2O for three times for 5 min. followed by 70% ethanol for 10 min. The explants were again washed with dH_2O three times and blotted dry on sterilized filter paper. Finally, ten sterilized explants were aseptically placed on tissue culture mediums.

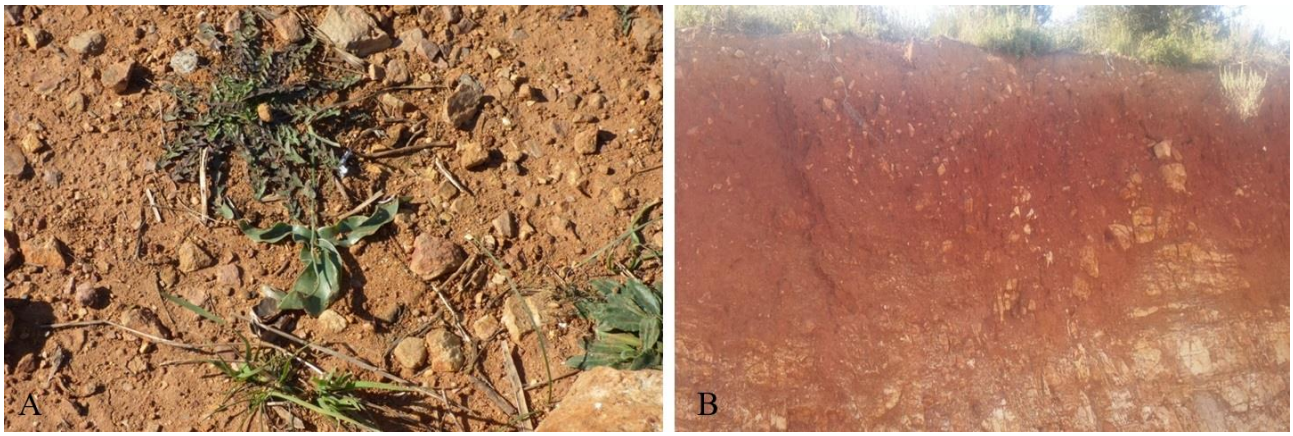

Fig. 1. The red and iron-rich Terra Rosa soil at Çamlıca Hill where *C. chalcedonicum* specimens were found during the study.

Fig. 2. The natural appearance of *C. chalcedonicum*' in field studies (A and B**)** and in The Istanbul University Alfred Heilbronn Botanical Garden (C and D).

Inoculation and Incubation

The disinfected explants were cultured on MS medium (Murashige & Skoog 1962) supplemented with different concentrations and combinations of sucrose and plant growth regulators (PGRs) [1-Naphthalene acetic acid (NAA), 6-Benzylaminopurine (BAP), Zeatin (ZEA), 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-(γ,γ-Dimethylallylamino) purine (2IP)]. To induce callus induction, all cultures were maintained in a growth chamber for two weeks to several months at 18-25 ± 2.0°C with dark according to used medium. After callus induction, the cultures were sub-cultured at 18-25 ± 2.0°C with the light intensity of 75 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps for a photoperiod of 16:8 hours of light and dark period in every 24-hour cycle. Relative humidity of the growth chamber was kept at 50%. Active carbon was also used for development of callus. MS medium was prepared with some modifications. The pH of the media was first adjusted to 5.8, then it was autoclaved for 15 min at 121°C. As the control group, MS medium supplemented with 2% Sucrose (Suc) were used. The explants were cultured on MS medium supplemented with 3, 8 and 10% (w/v) Suc and various plant growth regulators. For initiating callus, different treatment combinations including 2,4-D as an auxin (2 mg ml^{-1}) and BAP as a cytokinin (0.5 mg ml^{-1}); NAA (1 mg ml^{-1}) and ZEA (1 mg ml^{-1}); NAA (2 mg ml^{-1}) and BAP (0.5 mg ml^{-1}); 2,4 D (2 mg ml^{-1}) and BAP (0.5 mg ml^{-1}); 2,4 D (2 mg ml^{-1}) and ZEA (1 mg ml^{-1}); 2,4 D (2 mg ml^{-1}), ZEA (1 mg ml^{-1}) and BAP (0.5 mg ml^{-1}); 2,4 D (2 mg ml^{-1}) and 2IP (0.5 mg ml^{-1}) were applied to the corm slices as three replicates. The last medium was applied as 2^{-1} MS supplemented with 2,4 D (2 mg ml^{-1}), 2IP (0.5 mg ml^{-1}), 3% Suc and 0.05% active carbon according to the report of Yalcin Mendi *et al.* (2017).

Results

Various explant types, PGRs, different sugar concentrations, and chemicals were tested for callus induction in *C. chalcedonicum*. Callus were only formed from corms (see Fig. 3). Leaves and corm shells as explant demonstrated no development for callus formation. Different researchers used different sterilization protocols which included different concentrations and combinations of NaOCl, NaCl, $HgCl_2$, Tween 20 and Tween 80 (Khan *et al.* 2011, Daradkeh *et al.* 2012, Wagh *et al.* 2015). In our sterilization protocol, we observed 0.25% (w/v) mercuric chloride ($HgCl_2$) and 6.5% NaCl with Tween 80 were required to sterile *C. chalcedonicum* corms. However, NaOCl is not necessary for sterilization of *C. chalcedonicum* corms.

After determination of the sterilization protocol, different sucrose concentrations were tested. At the first step, two sucrose concentrations (3% and 8%) were tested for all different PGRs combinations. Corms on 8% sucrose were formed as green and soft callus. Callus formation in 3% sucrose was found to be slower than 8% sucrose, thus corms in 3% sucrose were transferred to

mediums containing 10% sucrose to enhance callus formation.

Callus development was observed within a week after transferring medium to MS supplemented with NAA (1 mg ml^{-1}), ZEA (1 mg ml^{-1}) and 10% Suc, becoming partly green and soft. Callus formation ratio in this media was around 15%. Additionally, callus formation was observed on 2^{-1} MS supplemented with 2,4 D (2 mg ml^{-1}), 2IP (0.5 mg ml^{-1}), 3% Suc and 0.05% active carbon (see in Fig. 4). In this medium, callus formation ratio was around 75%. However, some cultures were transferred to 2^{-1} MS supplemented with 2,4 D (2 mg ml^{-1}), 2IP (0.5 mg ml^{-1}), 3% Suc, 0.05% active carbon medium after twelve weeks and the corms were observed to get a green colour and swelling. Eventually, after testing different PGRs and sugar concentrations, callus formations were only observed on MS supplemented with NAA (1 mg ml^{-1}), ZEA (1 mg ml^{-1}) containing different concentration of sucrose -%8 and 10% Suc- and 2^{-1} MS supplemented with 2,4 D (2 mg ml^{-1}), 2IP (0.5 mg ml^{-1}), 3% sucrose, 0.05% active carbon medium.

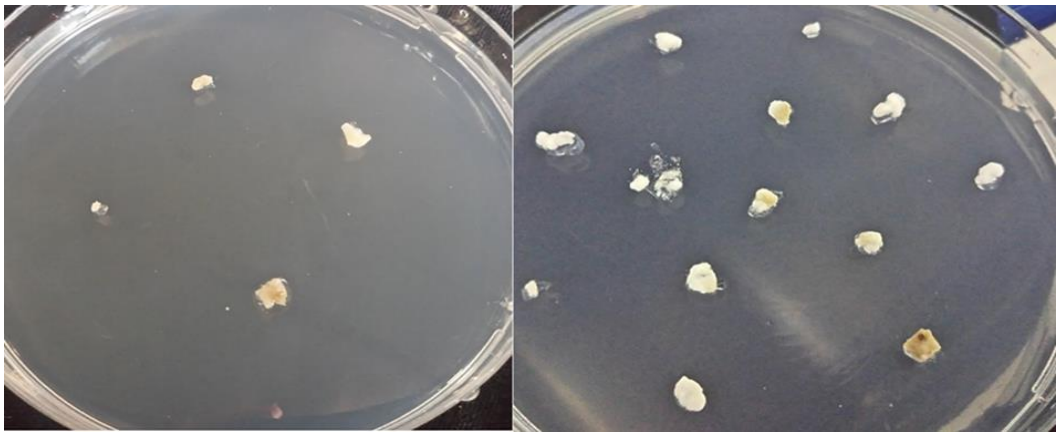

Fig. 3. Callus formation of *C. chalcedonicum* on [ZEA (1 mg ml^{-1}) + NAA (1mg ml^{-1}) + 10% Suc + MS basal medium]

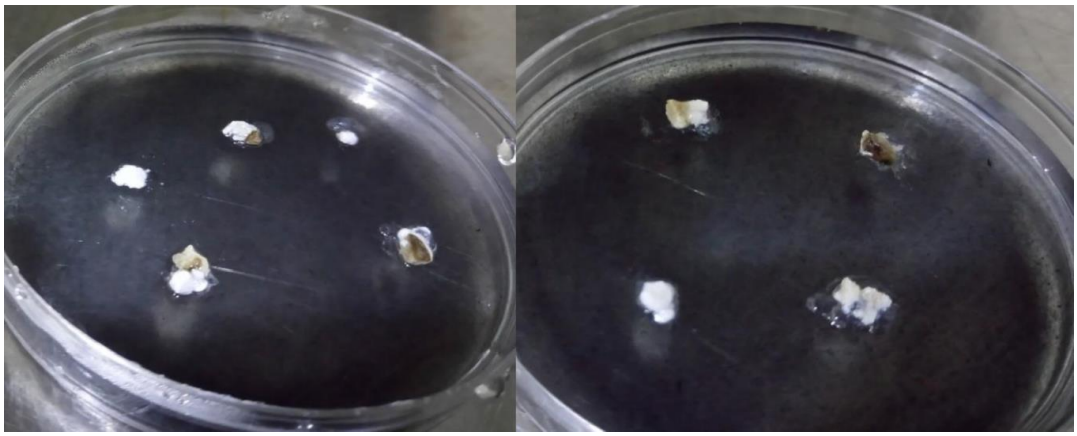

Fig. 4. Callus formation of *C. chalcedonicum* on [2,4-D (2 mg L^{-1}) + 2IP (0.5 mg L^{-1}) + 3% Suc + 0.05% active carbon + 2^{-1} MS basal medium]

Discussion

The genus *Colchicum* in Turkey has a wide distributional range with 50 species of which 15 are endemic for the country (Dahlgren *et al.* 1985, Persson 1999a, Akan & Eker 2005). In addition to the relatively wide distribution range for the genus, Turkey is the richest country in terms of the species diversity. However, in the present study, only 61 *C. chalcedonicum*, known to be grown on rich red soils at dry stony and rocky places, were found only at Çamlıca Hill which is formed from the red, iron-rich Terra Rossa soil (see Fig. 2).

Limited number of experiments were performed about *Colchicum* tissue culture (Hayashi *et al.* 1988, Yoshida *et al.* 1988, Khan *et al.* 2011, Daradkeh *et al.* 2012, Wagh *et al.* 2015). In our sterilization protocol, we used 0.25% (w/v) $HgCl_2$ for surface sterilization, while Wagh *et al.* (2015) used 0.15% (w/v) $HgCl_2$ for surface sterilization of *C. luteum* Baker corms. The reason for the use of 0.25% (w/v) $HgCl_2$ in our study was that the soil where the corm explants obtained from Çamlıca Hill is composed of mineral particles, organic matter, water, air and living organisms.

In our study, we tested different sucrose concentrations -(3, 8 and 10%) and 8% and 10% resulted with better callus formation. Nagaraju *et al.* (2002) tested 30, 60, 90 and 120 g L^{-1} sucrose concentrations and observed that sucrose showed a significant effect on corm size and weight, leaf

weight and root length. Additionally, studies revealed that *Colchicum* corms were more desirable for callus induction (Daradkeh *et al.* 2012, Wagh *et al.* 2015). Our study demonstrated that corm was better than leaves and corm shells as an explant for callus development. Callus formation rates for corm were observed around 15% and 75% for MS supplemented with NAA (1 mg ml^{-1}), ZEA (1 mg ml^{-1}) containing 10% sucrose- and 2^{-1} MS supplemented with 2,4 D (2 mg ml^{-1}), 2IP (0.5 mg ml^{-1}), 3% sucrose and 0.05% active carbon medium, respectively (see Figs 3, 4). However, no callus formation was observed from leaves and corm shells, indicating that they are not suitable for callus formation as explant. *Colchicum chalcedonicum* is known as a calcitrant species. Because of its advantages, micro-propagation of corm plants could be an alternative to the conventional techniques for vegetative propagation, increasing many times the multiplication level, enabling the plant materials to be freed from diseases especially for medicinal plants (Shibli & Ajlouni 2000, Chang *et al.* 2000).

Our efforts are continuing for developing callus formation protocol. Yalcin Mendi *et al.* (2017) reported micro-propagation of some endemic *Colchicum* species, but not on tissue culture for *C. chalcedonicum.* However, they used to active carbon to induce callus formation in *Colchicum* cultures. Active carbon is frequently used in tissue cultures to improve micro-propagation, orchid seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, rooting, stem elongation, corm formation etc (Thomas 2008). Studies with activated carbon in tissue culture demonstrated that activated carbon may be provide irreversible adsorption of inhibitory compounds in the culture medium significantly reducing the toxic metabolites, phenolic exudation and brown exudate accumulation (Thomas 2008). In our study, active carbon may enhance callus formation by limiting the brown exudate accumulation. We observed that green and soft callus formation resulted in medium containing active carbon. Also, callus formation in medium containing active carbon was faster than other mediums we tested.

Colchicum species comprise flavonoids, phenolic acids, tannin, fatty acids and colchicine is the major alkaloid isolated from *Colchicum* species such as *C. autumnale* and *C. luteum* (Kapadia *et al.* 1972, Levy *et al.* 1991, Evans 2002). Studies demonstrated that colchicine possesses antitumoral and anti-inflammatory activity and that it has a great potential for cancer treatment, making derivatives of colchicine, especially demecolcine and trimehyl colchicine acid methyl ester, to be evaluated as anti-cancer agent (Cocco *et al.* 2010, Bisi *et al.* 2015). However, no systematic attempt has been performed on micro-propagation for elite genotypes such as "Medicinal Plants" including *Colchicum* species. Our callus formation protocol has served promising results for future. Hayashi *et al.* (1988) used IBA and kinetin as PGRs in *C. autumnale* tissue culture for callus formation, and they managed to obtain colchicine by callus tissue culture system. However, production of secondary metabolites can be achieved by two main groups of *in vitro* cultures: organized cultures of differentiated tissues (i.e., organ cultures as root, shoot and embryo cultures) and unorganized cultures of undifferentiated cells (i.e., callus and cell suspension cultures). Although organized cultures of differentiated tissues produce the same secondary metabolites as the plant itself, which are relatively more stable than the undifferentiated cells, especially non-embryogenic plant callus cultures are mostly used for production of valuable secondary metabolites, including such as tropane alkaloids, hyoscyamine and scopolamine (Verpoorte *et al.* 2002, Filova 2014). Our present study is the pioneering study for tissue culture of *C. chalcedonicum.*, and tissue culture studies of *C. chalcedonicum* may also be applied for callus formation of important *Colchicum* species possessing colchicine.

An organism is identified as "endemic" which is native and has a restricted geographical region. Endemic species may be restricted due to physical barriers to dispersion, as in the case of many island faunas and flora, the barriers surround its area of origin, and consequently, they evolve within their limited distributional ranges (Masseti 2009). The extinction of plant and animal species, particularly with the ongoing climate change effects, has become an important issue, especially for endemic species. An alternative method of protection of endemic plant species is producing them *via* multiplying and conservation of plants by using *in vitro* culture techniques. The producing of endemic plants using tissue culture systems *via* multiplying is termed micro-propagation which has lots of advantages including high coefficient of multiplying, small needs on number of initial plants, small needs on space and reproducing of plants regardless seasons of the year, through multiplying intervals between subcultures in slow growing species (Kováč 1995, Engelmann 1997, 1998). We aimed to find the best nutrient media, PGRs and explants for micro-propagation and *in vitro* conservation of *C. chalcedonicum.* However, we were able to establish only efficient callus protocol were the pioneering of micro-propagation. This callus formation protocol will serve the improvement of tissue culture techniques to obtain more efficient callus formation protocols and to lead the micro-propagation of *C. chalcedonicum* in the future.

Conclusion

The success of efficient callus protocol improvement for *in vitro* conservation of *C. chalcedonicum* relies on the optimal choice of the explants, on the efficiency of the sterilization method, and on the establishment of an *in vitro* culture protocol for these calcitrant species for the beginning of aseptic proliferative cultures and on the optimal choice of nutrient media and PGRs. According to our experimental data, the optimal media for efficient callus formation of *C. chalcedonicum* were MS supplemented with NAA (1 mg ml-1), ZEA (1 mg ml-1) containing 10% sucrose— and 2-1 MS supplemented with

2,4 D (2 mg ml-1), 2IP (0.5 mg ml-1), 3% sucrose and 0.05% active carbon medium. Additionally, supplying of active carbon in the media induced the callus formation. Our work is the pioneering study to obtain sophisticated callus formation protocol for *in vitro* conservation of *C. chalcedonicum.* Our study may help to save *C. chalcedonicum* which is endemic in İstanbul, Turkey.

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