**Effect of plant growth regulators and dark stress on croton (*Codiaeum variegatum*) callus induction.**

**Abstract**

The croton (*Codiaeum variegatum*) plant, with its magnificent leaf colors, is a beautiful indoor and outdoor ornamental plant as well as its leaf extracts have many medicinal properties. Usually, crotons are propagated vegetatively. This process is slow in response and many parent plant stocks need to be maintained. Micropropagation is an alternative propagation method that can meet its high demand in a relatively short time. Callus culture is a tissue culture method that is used both in micropropagation and in the production of secondary metabolites. The purpose of this study is to determine the effects of different growth plant regulators and dark growth conditions on callus induction of croton (Codiaeum variegatum). MS medium with 3 % sucrose, 0,8 % agar and different combinations of kinetin, benzyl amino purine (BAP) and Naphthalene acetic acid (NAA) was used.The highest callus diameter was measured as 1.0 cm in M9 medium under both dark and light growth conditions. The maintained dark conditions had a significant effect on callus induction in M4 and M9 media. While callus formation was 22.2% in these nutrient media kept in day/night conditions, it was 94.44 % in dark conditions.

**Keywords:** Callus culture, MS, Kinetin, Callus weight, micropropagation

**İntroduction**

Croton(*Codiaeum variegatum*) is one of the beautiful indoor and outdoor plants belonging to the Euphorbiaceae family. It is native to several countries such as East India, Malaysia, Pacific Islands. It is a typical tropical plant and is mainly grown for its leaves. These are plants in the form of shrubs. It can be sized 60-120 cm. It can also form small trees in its homeland. It has very decorative leaves of many different colors and shapes. Young leaves are usually green, yellow, and red, later may be gold, cream, white, red, pink, purple, black, or brown. Besides the beauty of the leaves of the crotons, the leaf extracts are reported to have many medicinal properties including laxative, soothing antifungal, and anti-cancer activities. The plant is also a good natural source for the production of important secondary metabolites such as alkaloids, tropenes, and flavonoids (Maciel et al., 1998; Puebla et al., 2003; Simona et al., 2008). Various methods are used for the vegetative propagation of crotons, such as cuttings, grafting, seeds, and air layering(Bakheet et al,2018). However, with these methods the process is long and many parent plant stocks need to be maintained. The use of plant tissue culture methods in propagation enables high demand to be met in a relatively short time (Nasib,20008). There are many techniques used in ornamental plant micropropagation. It is accomplished by various means, i.e. by the propagation of shoots from different explants such as leaves, shoot tips, or axillary buds, or by direct formation of incidental shoots or somatic embryos from tissues, organs, or zygotic embryos (Rout et al.,2006). Callus culture is one of these micropropagation methods. Callus culture is a plant tissue culture technique used in the production of medical secondary products as well as micropropagation. A callus is an unorganized mass of cells. Callus cultures can differentiate into whole plants, if maintained under suitable growth media that differ from standard culture media generally containing high auxin concentrations or a combination of auxin and cytokinin (Efferth, 2019,). Also, some callus cultures demand dark growing conditions while others grow under certain day-night conditions (e.g., 16 h light, 8 h dark). Callus cultures generally grow at 25 ± 2 °C.

There are many factors affecting the success in plant tissue culture such as genotype, nutrient medium, growth conditions. Also, it has been revealed in many studies that the pre-treatments such as warm, cold, and keeping in the dark affect the success in a positive way (Shi et al.,2020).

In this study, the effects of different plant growth regulator balance and dark stress on callus formation croton were investigated.

**Material and method**

The study was carried out in the Tissue Culture Laboratory of Gaziosmanpaşa University, Faculty of Agriculture, Department of Horticulture. Young leaves of Croton (*Codiaeum variegatum sp.)* were used as explant.

**Explant Sterilization**

At first, explants were collected (leaf-stem) and washed completely under running water for 30 minutes, followed by 70% ethanol for 1 minute. Afterward, they were disinfected for 15 minutes with 20% commercial sodium hypochlorite and added a few drops of Tween-20. At the end, explants were washed 3 to 5 times with sterilized distilled water.

**Composition and preparation of nutrient media**

The disinfected leaves were cut in 2-3 mm size with the help of a scalpel and placed in the nutrient medium. MS (Murashige and Skoog, 1962) medium was used as the nutrient medium. Explants were individually transferred into 10 ml petri dish containing 10 ml of basal MS medium with 3 % sucrose, 0,8 % agar and different combinations of kinetin, benzyl amino purine (BAP) and Naphthalene acetic acid (NAA). MS medium with twelve combinations was used (Table 1).

Table 1. Murashige and skoog (MS) medium with twelve compositions of plant growth regulators

|  |  |  |  |
| --- | --- | --- | --- |
| Media codes | NAA mgL-1 | KİNETİN mgL-1 | BAP mgL-1 |
| M1 | 0,5 | 0,5 | - |
| M2 | 0,5 | 1,0 | - |
| M3 | 1,0 | 0,5 | - |
| M4 | 1,0 | 1,0 | - |
| M5 | 0,5 | - | 1,0 |
| M6 | 0,5 | - | 2,0 |
| M7 | 1,0 | - | 1,0 |
| M8 | 1,0 | - | 2,0 |
| M9 | - | 0,5 | 1,0 |
| M10 | - | 1,0 | 2,0 |
| M11 | - | 0,5 | 1,0 |
| M12 | - | 1,0 | 2,0 |

**Growth conditions:**

Cultures were maintained at 25 ±2°C in two different growth conditions as light and dark. The light condition group was transferred directly to the Air Conditioning-room with 25 ±2 oC temperature, 16 hours of light and 8 hours of darkness, and 3000 lux light intensity. The dark growth condition group was kept in the dark for 15 days in a growth chamber set at 25 ±2 oC, then transferred to the acclimatization room and waited for callus formation.

**Callus formation rate**

The percentage of callus formation was calculated 50 days after culture.

Callus formation (%) = (number of explants forming callus ÷ Total number of explants) × 100.

Callus fresh weight (mg) = Fresh weight of different calli cultures was recorded after 50 days of cultivation. Each treatment was made in triplicate.

Callus Diameter (cm): Callus diameter of different calli were recorded after 50 days of cultivation. Each treatment was made in triplicate.

**Data analysis**

The least significant difference (Duncan) at P<0.05 was used for means comparison. Statistical differences between the nutrient media were shown in lower case, whereas statistical differences between the growth conditions were shown in capital letters.

**Result and Discusion**

Callus induction was observed on the explants approximately 20 days after culture. The effect of plant growth regulators and maintaining the cultures in the dark on callus formation 50 days after culture in *Codiaeum variegatum* is presented in Fig 1, Fig 2, and Fig 3.

**The effect of dark growth conditions on the callus induction**

Looking at the results, waiting in the dark did not have a positive effect on callus induction by itself. However, when looking at the media and light interaction, the positive effect of keeping it in the dark was seen in M4 and M9 medium. The highest rate of callus formation (88.89%) for the M9 medium was in dark conditions (Fig 1).

**The effect of different plant growth regulators on callus formation in croton**

In the study, 3 plant growth regulators (kinetin, NAA, BAP) were added to the nutrient media in 12 different combinations. The highest callus diameter (1.0 cm) was observed in M9 (MS+0.5 mg/L Kinetin+1.0 mg/L BAP) medium (Fig 2). In callus weight, the best result (444.6 mg) was obtained from M9 (MS+0.5 mg/L Kinetin+1.0 mg/L BAP) medium (Fig 3). There was no callus formation in media M10, M11, and M12. The best results were obtained from media combined with NAA.

MS combinated with auxin and cytokinin was the main media for the in vitro propagation o croton in the published literature (Shibata et al., 1996; Orlikowska et al., 2000; Nasib,20008). In our study, callus formation rates ranged from 0% to 100%. It was determined that M10, M11 and M12 media were not suitable for callus culture in croton. This showed that the high rate of cytokinin in nutrient medium negatively affects the induction of callus.

Our results indicated that M8 (1.0 mg/L NAA+2,0 mg/L BAP) and M9 (0,5 mg/L Kinetin+1,0 mg/L BAP), is the optimal medium for the callus weight of the *Codiaeum variegatum sp.*

Each callus cultures need diffrent growth conditions. While Some callus culteres want day-nigt conditions others grow under dark conditions. Many studies have indicated that incubation of cultures in dark conditions has a positive effect on plant regeneration (Kuehnle et al, 1992; Rutkowska-Krause et al,2003;Gao et al,2011).

It was significantly effective to keep the explants planted in M4 medium in the dark.

As a result, callus formation rates vary between 0% and 100%. The highest rate of callus formation was observed in M2, M6, M7, M8 environments. The highest callus diameter was measured in M9 medium (1 cm). Callus weight varies between 29.80 and 444.60 mg. It was determined that the medium prepared with NAA was more successful than the other mediums in terms of plant growth regulators.

Fig 1. Callus induction

Fig 2. Callus diameter (cm)

Fig 3. Callus weight (mg)

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