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Effects of Plant Growth Regulators on Callus Formation in Different Explant of *Calendula officinalis* L.

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Abstract
This study has been conducted to investigate different explants of plant growth regulators on callus induction of *Calendula officinalis* L. medicinal and aromatic herb. The seeds were surface sterilized using 70% ethanol for 3 minutes, 10% commercial bleach for 5 minutes and rinsed 3 times with sterile water for 3 minutes. The seeds were germinated in Murashige and Skoog (MS) medium. Hypocotyl, cotyledon and cotyledon node explants have been excised from the plantlets obtained from *in vitro* germinated seeds. Three explants were cultured on MS media supplemented with various concentrations of cytokinin (BAP; 1.0, 2.0 mgL^{-1}) and auxin (IBA; 0.1, 0,5 mgL^{-1}) for callus induction. At the end of eight weeks, the best results were observed in a treatment with 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA and 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 100% on the hypocotyl; 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA and 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 88% on the cotyledon; 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 100% on the cotyledon nodes. The data obtained from the path is instructive to gene transformation, phytoremediation and cell culture study.
Keywords: *Calendula officinalis* L., Cotyledon, Cotyledon node, Hypocotyl, Plant growth regulators.

INTRODUCTION

Plants have been used for important medicinal constituent in indigenous medical systems since ancient times. A large proportion of the drugs used in modern medicine is either directly isolated from plants or synthetically modified from a lead compound of natural origin. In plants many different types of organic compounds or metabolites are produced as a result of metabolic processes [1].

Calendula officinalis L., known for its ornamental plant characteristics, is a medicinal plant belonging to Asteracea family. It is a yearly or perennial taproot plant with 20-40 cm height and has 20 varieties [2]. As a result of the studies conducted with Calendula, many studies have reported the plant to have pharmacological effects such as anti-cancer [3, 4, 5, 6], anti-microbial [7, 8, 9, 10, 11], anti-leishmanial [12, 13], anti-HIV [14], antioxidants [15, 16, 17], cytotoxic, anti-tumor [18, 3, 19], anti-viral [20], anti-inflamatuar [21, 19], oedema diuretic [22], hypoglycemic [23], uterotonic [24], lymphocyte activator effect [3], in venous ulcer treatment [25] and for biligenic function [26].

In recent years, plant cell, tissue and organ culture technology has been efficiently utilized in the production of secondary metabolites [27]. Plant cell and tissue cultures can be performed routinely under sterile conditions from explants, such as plant leaves, stems, roots, and meristems for multiplication and extraction of secondary metabolites. Strain improvement methods for the selection of high-producing cell lines and medium optimizations can lead to an enhancement of secondary metabolite production. The capacity for plant cell, tissue and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. Many investigators have reported production of useful compounds in both callus and suspension cultures. Some secondary metabolites have been observed in much higher concentrations in cultured cells than in whole plants of the same species [28]. The strong and growing demand in today's marketplace for natural and renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products and has paved the way for new research exploring secondary product expression *in vitro* [29].

The callus are cell clusters in an undifferentiated state, which can be induced to a cell re-differentiation process through the addition of vegetal growth regulators, as well as to produce secondary metabolites of industrial interest. The requirements of plant growth regulators and their concentrations can influence the more viable callus, callus growth and could induce changes in the metabolism during the callogenesis phase [30].

In this study, the influence of BAP and IAA plant growth regulators administered to *Calendula officinallis* L. hypocotyl, cotyledon and cotyledon node explants, on callus induction has been studied.

MATERIALS AND METHODS

Experimental plant

Calendula officinalis L. seeds are provided from the Hekim Sinan Medicinal Plants Research Center of the Municipality of Kütahya.

Seed sterilization

C. officinalis seeds were washed under tap water and the seed coats were removed before sterilization. The seeds were surface sterilized using 70% ethanol for 3 mins, 10% commercial bleach (NaOCl at 5% v/v) for 5 mins, followed by three washes for 3 mins in sterile distilled water. Seeds were then germinated on half strength hormone-free MS (Murashige and Skoog, 1962) medium.

Callus induction

Hypocotyl, cotyledon and cotyledon node explants were excised from 5 weeks old *in vitro* grown seedlings. Callus initiation medium was supplemented with BAP (1 and 2 mgL^{-1}) separately and IBA (0.1 and 0.5 mgL^{-1}) in combination. Hypocotyl and cotyledon node explants were subcultured in 2 weeks and cotyledon explants were subcultured with 3 week intervals on the same medium for callus proliferation.

***In vitro* culture condition**

The basal MS medium used was supplemented with 100 mgL^{-1}(w/v) myo-inositol and 3% (w/v) sucrose. The pH of all types of media was adjusted to 5.8 before the addition of 0.7% agar. All chemicals used were of analytical grade (Sigma and Merck). The culture vials containing media were autoclaved at 20 psi, at 121^0C for 15 mins. Cultures were maintained in a plant growing room at 24±5 ^{0}C, under a 16 h photoperiod by cool white fluorescent tubes (Philips).

RESULTS

The length of plantlets which have been germinated in MS media for two weeks reached 2 to 5 cm and the percentage of germination was determined as 46%.

It was also determined that different plant growth regulators have no significant statistical importance on the callus induction percentage of hypocotyl explants of *C. officinalis* ($F=0.546$, $p>0.05$). Callus induction rates of hypocotyl explants treated with plant growth regulators were determined as 1 mgL^{-1} BAP 63%, 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 88%, 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 100%, 2 mgL^{-1} BAP 75%, 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 71%, 2 mgL^{-1} BAP + 0.5 IBA mgL^{-1} 100%. On the other hand, there are noticeable distinctions of colour and tissue on callus inducted by plant growth regulators which have been administrated to hypocotyl explants. Shoot regeneration from callus was observed in MS media containing 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA (Figure 1. and Table 1.).

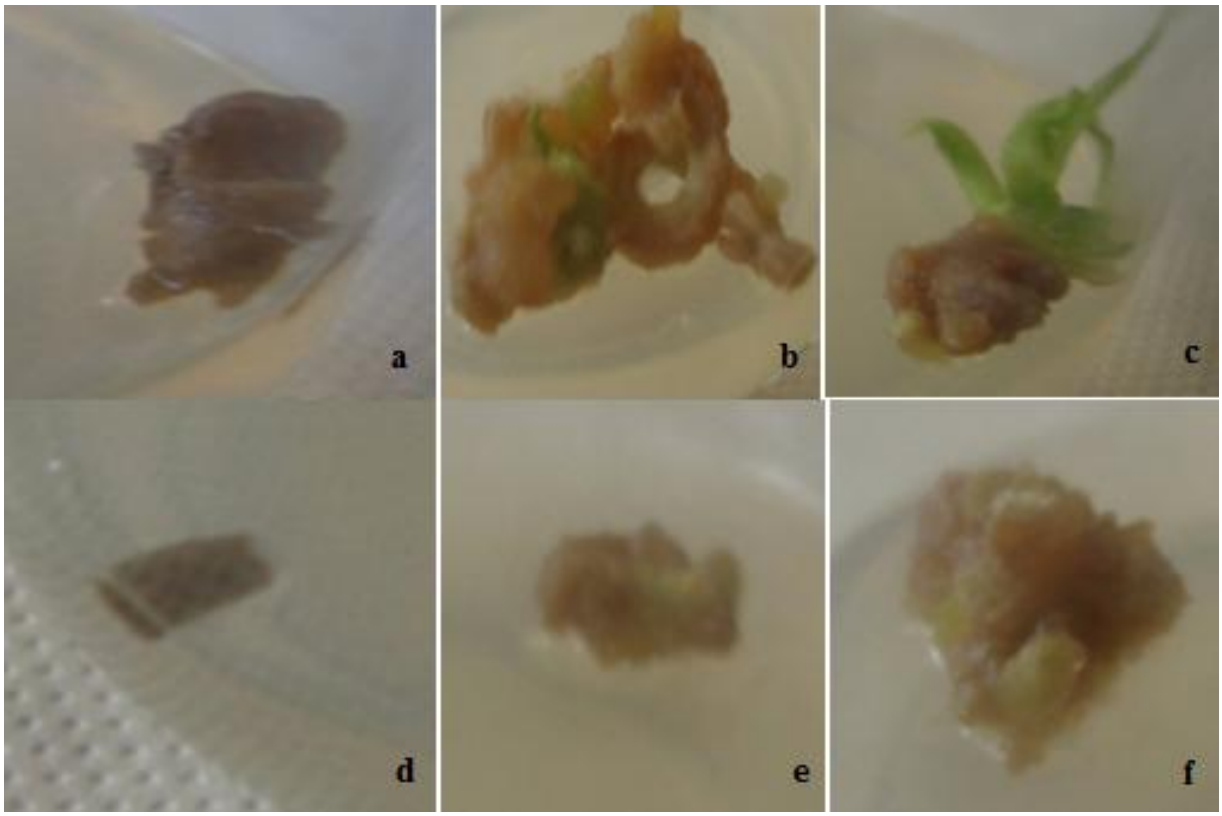

Figure 1. Callus formation on hypocotyl explants after 7 weeks
a) 1 mgL^{-1} BAP, b) 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, c) 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, d) 2 mgL^{-1} BAP, e) 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, f) 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA.

Table 1. Effect of various plant growth regulators on callus level, colour and texture of hypocotyl explants of *C. officinalis.*

Plant Growth Regulators (mgL^{-1})	Callus induction Level	Callus Color	Callus Texture
1 BAP	++	Greenish brown	Firm
1 BAP + 0.1 IBA	++++	Green + yellow + brown	Brittle
1 BAP + 0.5 IBA	+++	Brown	Firm and brittle
2 BAP	+	-	-
2 BAP + 0.1 IBA	++	Brown	Mellow and solid
2 BAP + 0.5 IBA	++++	Brown	Mellow and large

* + very few calluses , ++ few calluses , +++ a lot of calluses ++++ very good callus

It was also determined that different plant growth regulators have no significant statistical importance on the callus induction percentage of cotyledon explants of *C. officinalis* (F= 1.113 p>0.05). Callus induction rates of cotyledon explants treated with plant growth regulators were determined as 1 mgL^{-1} BAP % 0, 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 41%, 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 88%, 2 mgL^{-1} BAP 33%, 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 21%, 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 88%. In nutrient media containing few or no auxin, darkening has been observed in cotyledon explants. Indirect adventitious shoot formation was observed from cotyledon explants containing 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA of plant growth regulators. On the other hand, direct adventitious shoot formation was observed in 2 mg/L BAP treatment, without callus stage (Figure 2.)

It was also determined that different plant growth regulators have significant statistical importance on the callus induction percentage of cotyledon node explants of *C. officinalis* (F = 3.818, p<0.05). The highest callus induction rate was determined in 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA MS media. Callus induction rates were determined on basal parts of cotyledon node explants treated with plant growth regulators as 1 mgL^{-1} BAP 63%, 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 50%, 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 75%, 2 mgL^{-1} BAP 25%, 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA 100% ve 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 88% (Figure 3).

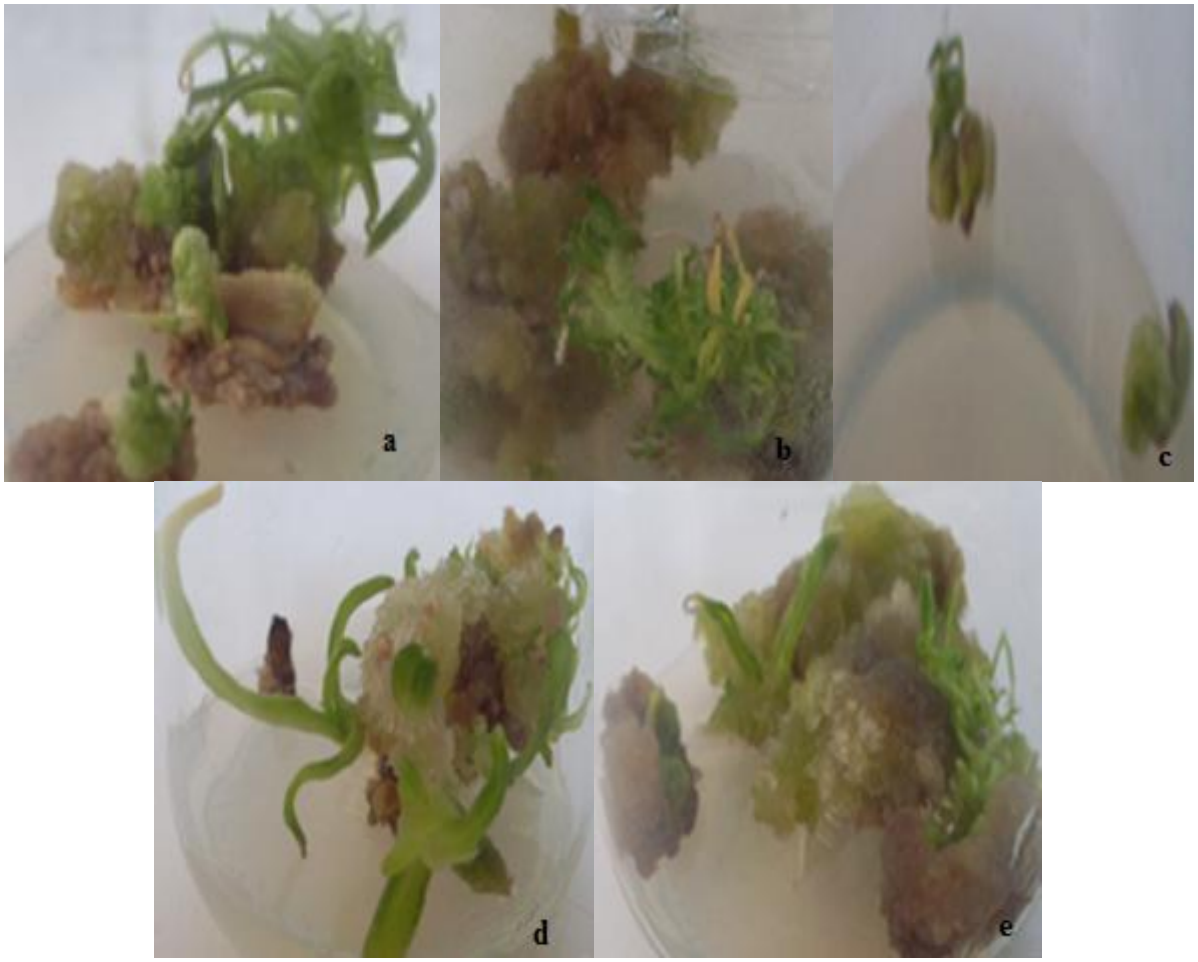

Figure 2. Callus and adventitious shoot formation on cotyledon explants after 15 weeks
a)1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, b) 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, c) 2 mgL^{-1} BAP, d) 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, e) 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 15 weeks.

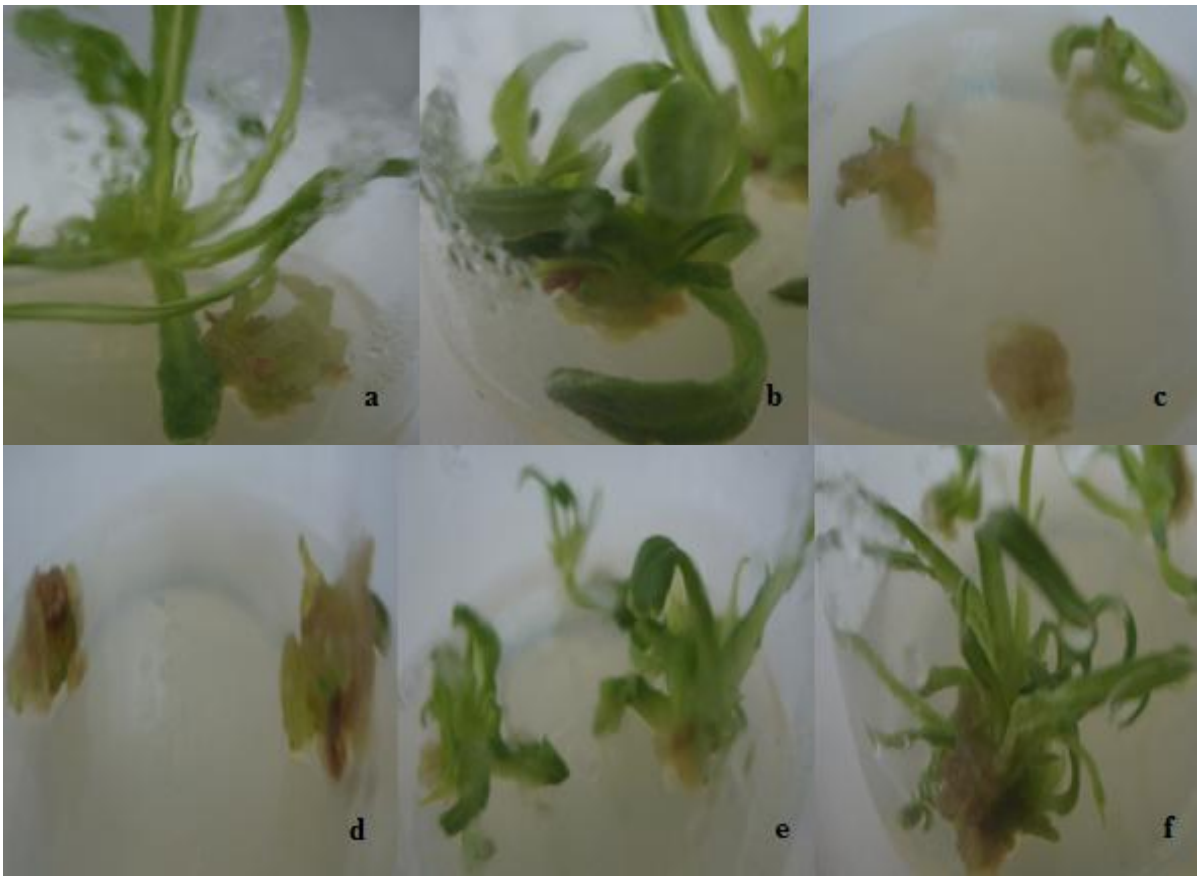

Figure 3. Callus and adventitious shoot formation on cotyledon node explants after 15 weeks
a) 1 mgL^{-1} BAP, b) 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, c) 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, d) 2 mgL^{-1} BAP, e) 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, f) 2 mgL^{-1} + 0.5 mgL^{-1} 15 weeks.

DISCUSSION

In tissue culture studies conducted in aseptic conditions, in order to minimize the contamination problem caused by the mother plant, explants are more appropriate to be obtained from plants grown in a controlled and sterilized environment rather than from an external environment. Furthermore, plants grown in a tissue culture environment, tend to regenerate easier and more quickly compared to the ones in nature [31]. As a consequence of the results obtained above, explants that will be used in studies are derived from plants obtained by means of surface-sterilized seeds germinated in MS media. Seeds are subjected to surface sterilization process by being kept for 3 minutes in 70% ethyl alcohol, for 5 minutes in 10% laundry bleach and for 3 minutes and 3 times in sterilized distilled water. Seed germination ratio was determined as 46%. As a consequence of surface sterilization process, 30% rate of contamination is observed. As the ratio of germination could be reduced, we did not apply prolonged or powerful disinfectants.

Callus inductions were observed in all hypocotyl explants. Callus induction ratios were detected as the lowest at 1 mgL^{-1} BAP 63% and the highest at 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 100% and 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA 100%. In calluses developed out of hypocotyl in nutrient media treated with 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, shoot formations were observed. Çöçü and his colleagues used the same plant in MS media containing 1 mgL^{-1} Kin + 0.5 mgL^{-1} NAA and obtained a shoot formation at a rate of 73%, in MS media containing 2 mgL^{-1} Kin + 0.5 mgL^{-1} NAA and obtained shoot formation at a rate of 67% [32]. These two results are very similar to the cytokinin and auxin concentrations of our study.

According to plant growth regulators applied in cotyledon explants, the lowest callus formation was in 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA at rate of 21% and the highest callus formation was in 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA at a rate of 88%. In media treated with 1 mgL^{-1} BAP, no callus inductions were detected due to darkening. Shoot regeneration was determined on callus derived from cotyledon explants in media treated with plant growth regulator 1 mgL^{-1} BAP + 0.1 mgL^{-1} IBA, 1 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, 2 mgL^{-1} BAP + 0.1 mgL^{-1} IBA ve 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA. Direct organogenesis was observed in 2 mgL^{-1} BAP MS media. Auxin and cytokinin play an important role in organ differentiation in plant tissue culture. While high cytokinin/auxin ratio supports shoot formation, high auxin/cytokinin ratio supports root formation [33]. In our study, too, shoot formations are observed in media treated with high cytokinin concentration. Çöçü et all. obtained shoots on cotyledon explants in MS media treated with 1 mgL^{-1} Kin + 0.5 mgL^{-1} NAA, 2 mgL^{-1} Kin + 0.5 mgL^{-1} NAA. When compared with the results of the researchers mentioned above, the plant growth regulator BAP used as cytokinin triggers more shoot formation. However, we observed that in media with stabilized cytokinin concentrations but increased auxin concentrations, the number of callus inductions increased.

Callus inductions in cotyledon node explants were detected to be the lowest in media treated with 2 mgL^{-1} BAP 25%, the highest with 2 mgL^{-1} BAP + 0,1 mgL^{-1} IBA %100. Plant growth regulators applied in numerous studies conducted by different researchers using *C. officinallis* were reported to lead to callus inductions of a high ratio [34, 32]. In spite of the callus formation on the bazal parts of cotyledon node explants, the best shoot growth was determined containing 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA MS media and the longest shoot containing 1 mgL^{-1} BAP MS media. In the study conducted by Kim and his colleagues on *Echinacea angustifolia*, the best shoot growth was observed in media treated with 2 mgL^{-1} BAP + 0.5 mgL^{-1} IBA, and these findings seem to support the results of our study [35].

In recent years, secondary metabolite production using callus cultures and studies on the biological roles of these metabolites have gained speed. [36,37,38,39,40]. The amount of secondary metabolites obtained from callus cultures is different both in the source of explants and in calluses differentiating into shoot or root. Our study indicates the induction percentage and differentiation patterns of calluses formed by the influence of plant growth regulators applied to different explant types. The results of our study shed light to future studies on secondary metabolites that will be obtained from plant calluses.

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