The effect of different concentrations of TDZ and BA on *in vitro* regeneration of Iranian cannabis (*Cannabis sativa*) using cotyledon and epicotyl explants

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ABSTRACT: The present study was carried out to investigate micropropagation possibility and determine the optimal medium composition and plant growth regulators (PGRs) combinations under *in vitro* conditions in Iranian cannabis. The cotyledon and epicotyl explants obtained from 1 month old *in vitro* grown seedlings were used in MS medium containing BA (0.1, 0.2, 0.5, 1, 2 and 3 mg^{-1}) and TDZ (0.1, 0.2, 0.5, 1, 2 and 3 mg^{-1}) either alone or in combination with 0.5 mg^{-1} IBA. Callus formation had priority over direct regeneration in most of the PGRs treatments. Comparing the two explants, cotyledon had higher callus formation frequency and the largest callus volume was obtained for this explant in MS medium supplemented with 3 mg^{-1} TDZ + 0.5 mg^{-1} IBA. The highest callus fresh weight (3.15 gr) was obtained for cotyledon explant treated with 2 mg^{-1} TDZ+ 0.5 mg-1 IBA. In shoot formation step, the highest rate of shoot regeneration was achieved in the calli produced from epicotyl explant treated with 2 mg^{-1} BA + 0.5 mg^{-1} IBA; and the highest length of regenerated shoots (1.23 cm) was observed in 2 mg^{-1} BA + 0.5 mg^{-1} IBA treatment. In general, cotyledon was the best explant and TDZ in combination with IBA was the best treatment for callus formation. Epicotyl explant also showed better regeneration compared to cotyledon.

KEYWORDS: Cannabis, Callus, Plant growth regulators, Regeneration

INTRODUCTION

Cannabis (*cannabis sativa*) is a dicotyledonous plant which belongs to cannabinaceae family. Historical evidence shows that the plant has been used since old times as medicine, fiber, textile, etc. (12, 22). It is an annual dioecious plant that can grow up to 1-5 meters based on genetic and environmental condition (16). The crop is a real factory for the production of secondary metabolites that produces valuable compounds such as alkaloids, flavonoids, lignins and phenolics (4). Terpenes are another group of compounds that found in cannabis highly, causing the characteristic odor of the crop (7). Cannabis harbors more than 421 chemicals among which, 61 compounds are classified as Cannabinoids (2, 13). Considering medicinal uses, cannabis has been used as an appetite stimulant, antiemetic and anti-vomiting, anti-spasm (13), anti-epilepsy, anti-glaucoma and anti-asthma (2, 8).

Regarding cross-pollination nature of cannabis, retention and propagation of THC-rich pure genotypes of the crop are difficult and superior genotypes can't be propagated

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by seeds. Modern biotechnological approaches including tissue culture can effectively facilitate propagation of such medicinal plants. Actually there are few reports on direct and indirect regeneration of cannabis in culture medium and existing documents imply recalcitrant nature of this plant to regeneration. Current literature suggests that callus induction is an easy practice in this plant; while shoot regeneration from callus is always accompanied with much difficulty (4, 17). To regenerate cannabis plantlets, various explants prepared from different developmental stages of maternal plant have been used in culture media containing various concentrations of plant growth regulators. However, the reports on this topic are highly contradictory. Direct regeneration and regeneration via callus have only been reported in few papers (5, 10, 11). Most of the studies have dealt with development of cell suspension systems for achieving secondary metabolites containing in this plant. Shoot regeneration from shoot tip explant in MS medium containing 0.2 mg^{-1} of TDZ (21), shoot regeneration from node and axillary buds in MS medium supplemented with BA, Kin, TDZ and GA_3 (10), callus induction and regeneration from explants including leaf, petiole, node and apical bud on MS medium supplemented with various plant growth regulators including Kin, Dichamba, 2,4-D and NAA (20), and plant regeneration from calli of leaf explant on MS medium containing various concentrations of IBA, IAA, 2,4-D and NAA combined with 0.5µM of TDZ have been reported as some examples in this regard (11). Iran has a diverse four season climate with high potential growing this valuable medicinal plant. Although cannabis is a native plant in Iran, there has been no report on regeneration and micro-propagation of this valuable crop via tissue culture techniques. Moreover, there is no report on application of cotyledon and epicotyl as explants for micro-propagation of cannabis at the global level. The present study was conducted to optimize *in vitro* callus induction and regeneration of cannabis using cotyledon and epicotyl explants on culture media containing various concentrations of BA and TDZ individually or in combination with IBA in order to investigate the efficiency of these two explants and BA and TDZ for callus induction and regeneration of cannabis for the first time.

MATERIALS AND METHODS

For surface disinfection, seeds were rinsed with tap water, placed at 70% alcohol for 30 seconds, and then washed with sterile water three times. Then, surface disinfection was performed using 2% sodium hypochlorite together with 1 drop of tween 20 for 20min and finally, the seeds were thoroughly rinsed with water four times. Next, seeds were disinfected for 5 min in $Hgcl_2$ 0.05% and finally were washed with sterile distillated water. The seeds were cultured on hormone-free MS medium to germinate and produce seedlings for obtaining explants. Also, Explants including cotyledon and epicotyl were excised from *in vitro* grown plants after 30 days and transferred to media containing TDZ and BA individually or in combination with IBA (Table 1).

Subculture of explants was repeated every 30 days; and after two months, calli volume, fresh and dry weight of calli, the number of regenerated shoots, lengths of the regenerated shoots, length and number of roots formed on the calli were measured and recorded. To induce rooting, regenerated shoots were transferred to MS media containing NAA and IBA in four concentrations (0.1, 0.2, 0.5 and 1 mg^{-1}).

The number and length of roots were investigated after three weeks. Rooted shoots, after washing the roots with distillated water, were cultivated in pots containing equal ratio of perlite and pit moss. To avoid evaporation, the pots were covered with a transparent cover and placed in growth chamber under 25°C, 16/8 light and darkness photoperiod. The seedlings were regularly irrigated.

The covers were also removed after two weeks and plants were transferred to the greenhouse. A factorial experiment based on completely randomized design was used in four replications.

Data were analyzed using SAS software and mean comparison was conducted by MSTAT-C software; data normality was tested before conducting ANOVA. Furthermore, multiple ranges Duncan test was used for mean comparison (3).

RESULTS

Effects of various PGRs combinations on callus formation

In this experiment, the response of epicotyl and cotyledon explants cultured on MS basal medium containing 24 different combinations of plant growth regulators was investigated (Table 1). Surprisingly, it was observed that in contrast to most of plant species in which these PGRs combinations result in direct regeneration, the situation was completely different for cannabis explants, meaning that callus formation was dominant over direct

regeneration. The first response of explant to callus formation was observed after 11 days and callus formation was induced in both of the explants. The induced calli were different from each other regarding quality, shape, color, size and fresh and dry weight.

Effects of BA and IBA on callus induction

According to ANOVA results, single effect of BA or its interaction with IBA was significant regarding the callus size (p<0.01). The interaction of hormone concentrations and explants type was also significant (p<0.05). Moreover, a significant difference was observed between the explants investigated in the present study (Table 2).

Explants response to various concentrations of BA and IBA was different, so that the highest (4.68cm^3) and the lowest (0.03cm^3) callus size was obtained in 2 mg^{-1} BA and 0.5 mg^{-1} IBA in cotyledon tissue and 0.1 mg^{-1} BA in the same tissue, respectively (Figure 1). By increase in BA concentration from 0.1 to 2 mg^{-1}, the mass of callus produced in both explants was promoted, but in 3 mg^{-1} BA the callus mass was reduced. The addition of IBA in various concentrations of BA had positive influence on callus induction; so that callus volume was enhanced by the addition of this hormone to various levels of BA hormone. In general, cotyledon explant showed better response compared to epicotyl explants regarding the mass and size of the calli produced in various hormonal combinations (Figure 1).

Single effect of BA or its interaction with IBA on callus fresh weight in the two explants was significant (p<0.01); however, there was no significant difference between the explants (Table 2). In MS medium containing only BA or BA and IBA, the highest callus fresh weight was obtained in 2 mg^{-1} BA in combination with 0.5 mg^{-1} IBA for cotyledon explant; whereas the lowest amount was obtained in 0.1 mg^{-1} BA in cotyledon explant (Figure 2). Regarding dry weight of calli produced by the explants, there was no significant difference between the explants.

Table 1. Various combinations of PGRs used for callus induction (mg/l).

BA	IBA	TDZ	IBA
0.1	0	0.1	0
0.2	0	0.2	0
0.5	0	0.5	0
1	0	1	0
2	0	2	0
3	0	3	0
0.1	0.5	0.1	0.5
0.2	0.5	0.2	0.5
0.5	0.5	0.5	0.5
1	0.5	1	0.5
2	0.5	2	0.5
3	0.5	3	0.5

Effect of TDZ and IBA treatments on callus induction

Single effect of TDZ or its interaction with IBA on callus mass in the two explants was significant (p<0.01); moreover, the interaction of the plant growth regulators and explant type was also significant (p<0.05). Furthermore, a significant difference was observed between the explants (Table 3).

In response to TDZ alone or in combination with IBA, the highest(6.5cm^3) callus induction was observed in MS medium containing 3 mg^{-1} TDZ and 0.5 mg^{-1} IBA in cotyledon explant; while the lowest (0.13 cm^3) callus induction amount was observed in 0.1 mg^{-1} TDZ with the same explant (Figure 4). No callus induction was observed for the explants cultured in MS medium containing 0.5 mg^{-1} TDZ + 0.5 mg^{-1} IBA.

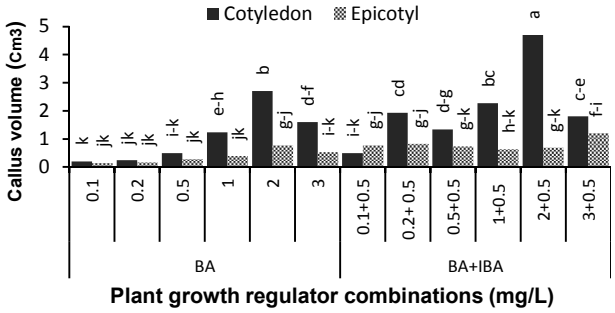

Figure1. Effect of various combinations of BA and IBA on callus volume (mm3) in cotyledon and epicotyl explants. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

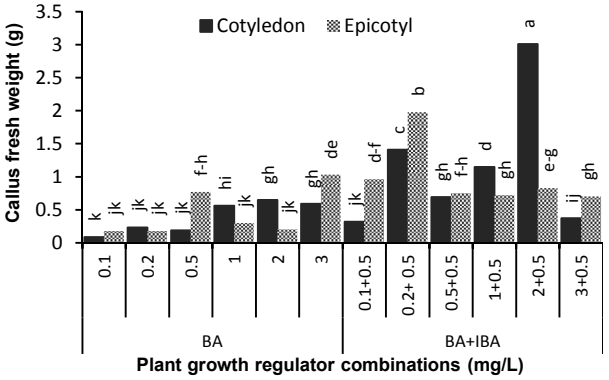

Figure 2. Effect of various BA combinations on callus fresh weight (mg) in cotyledon and epicotyl explants. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

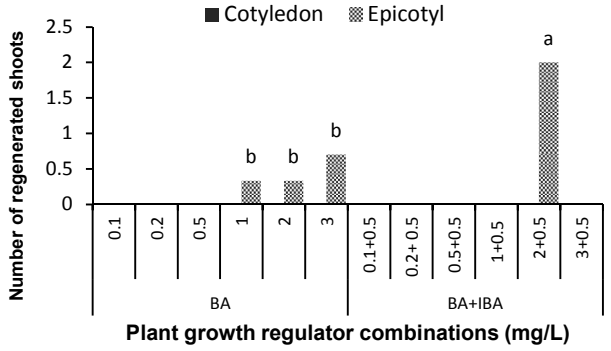

Figure 3. Effect of various combinations of BA and IBA on the number of the regenerated shoots. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

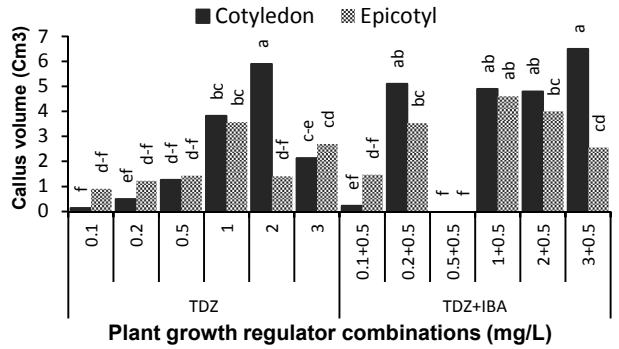

Figure 4. Effect of various combinations of TDZ and IBA on callus volume in epicotyl and cotyledon explants. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

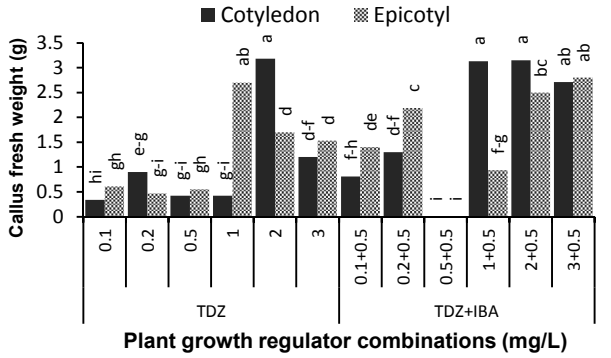

Figure 5. Effect of various combinations of TDZ and IBA on callus fresh weight in epicotyl and cotyledon explants. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

Table 2. Analysis of variation for callus induction traits in different explants and PGRs combinations.

PGR combination	Df	Mean Squares	
		Callus volume	Callus fresh weight
BA	5	4.92**	0.93**
IBA	1	9.81**	7.82**
Explant (E)	1	17.23**	0.1ns
BA* IBA	5	0.22ns	1.49**
BA* Explant	5	3.51**	1.37**
IBA* Explant	1	1.77*	0.24*
BA* IBA* E	5	0.58*	0.55**
Error	48	0.13	0.016

*, **: Significant difference at 0.05% and 0.0.1% probability level.

In cotyledon explant, by increase in TDZ concentration from 0.1 to 2 mg^{-1}, callus mass was enhanced and then reduced in 3 mg^{-1} mg^{-1}. However, in epicotyl explant by increase in TDZ concentration from 0.1 to 1 mg^{-1} callus mass was increased and then decreased at 2 mg^{-1} TDZ and again increased at 3 mg^{-1}. The addition of IBA to various concentrations of TDZ had a positive effect on callus induction and callus volume.

According to ANOVA results, the single effect of TDZ or in combination with IBA had a significant effect on callus fresh weight (p<0.01), but there was no significant difference between the explants regarding this trait (Table 3). Among various hormonal combinations, the highest callus fresh weight was observed in MS medium containing 2 mg^{-1} TDZ, 1 mg^{-1} TDZ combined with 0.5 mg^{-1} IBA, and 2 mg^{-1} TDZ combined with 0.5 mg^{-1} IBA in cotyledon explant; while the lowest callus fresh weight was obtained in MS medium containing 0.1 mg^{-1} TDZ in the same explant (Figure 5). Regarding callus dry weight, there was no significant difference between the explants in various concentrations of hormones. No statistical analysis was performed on callus color and fragility; however, based on the observations and recorded notes, it was evident that explant regeneration was determined by callus quality. In general, calli produced by MS medium containing TDZ hormone had better quality than those produced by BA.

Effect of growth regulators used for callus induction on shoot and root induction

In the present study, no direct regeneration was achieved under hormonal treatments; rather, only callus induction was observed.

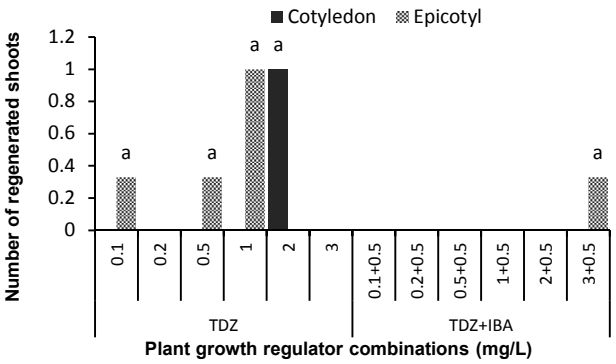

Figure 6. Effect of various combinations of TDZ and IBA on the number of the regenerated shoots. Each bar represents the mean of 20 replications. Bars indicated by similar letters are not significantly different at P=0.05 (Duncan's new multiple-range test).

Table 3. Analysis of variation for callus induction traits in different explants and PGRs combinations.

PGR combination	Df	Mean Squares	
		Callus volume	Callus fresh weight
TDZ	5	30.68**	9.20**
IBA	1	20.12**	5.94**
Explant (E)	1	7.97*	0.01ns
TDZ* IBA	5	7.96**	1.22**
TDZ* E	5	5.26*	0.85**
IBA* Explant	1	0.98ns	0.69*
TDZ* IBA* E	5	5.70*	3.22**
Error	48	1.06	0.1

*, **: Significant difference at 0.05% and 0.0.1% probability level.

Regarding media containing various concentrations of BA, alone or in combination with IBA, shoot induction was only obtained in epicotyl explants and cotyledon explants showed no response of shooting (Figure 3). Regeneration was observed in both cotyledon and epicotyl explants under treatments with TDZ and IBA (Figure 6). Based on ANOVA results, the effect of BA hormone, alone or in combination with IBA, on the number of regenerated shoots was significant (p<0.01); however, the effect of TDZ hormone, alone or in combination with IBA, on the number of regenerated shoots was not significant (p<0.01). The highest shoot regeneration rate was achieved in epicotyl explants cultured on MS medium containing 2 mg^{-1} BA in combination with 0.5 mg^{-1} IBA. Regarding the shoot length index, 2 mg^{-1} BA + 0.5 mg^{-1} IBA was superior to other treatments. Moreover, calli produced at various BA concentrations had rooting ability; however, no rooting was observed in the calli under TDZ treatments (results not shown).

Root induction in the regenerated shoots

Ten weeks after culturing the regenerated shoots on MS media supplemented with four concentrations of NAA and IBA (0.1, 0.2, 0.5 and 1 mg^{-1}), the highest root induction was observed in MS media containing IBA hormones. By increase in IBA concentration, rooting rate of the regenerated shoots decreased and completely stopped after a while. Various concentrations of NAA had a similar effect on root induction in the regenerated shoots and there was no significant difference among NAA levels. Moreover, burning was observed in the shoots cultured in media supplemented with NAA hormone. In addition, roots induced in IBA treatment were longer and thinner than those produced in media containing NAA hormone. The highest rooting rate in the regenerated shoots was obtained in MS medium containing 0.1 mg^{-1} IBA. Root length index was also investigated under auxin treatments and the longest root was formed in MS medium containing 0.5 mg^{-1} NAA. The regenerated seedlings were transferred to greenhouse after adaptation in phytotron. About 70% of the seedlings produced in tissue culture conditions survived and showed normal growth.

DISCUSSION

For *in vitro* regeneration of cannabis plants, epicotyl and cotyledon explants were cultured in MS medium supplemented with various combinations of BA, TDZ and IBA hormones. With one exception, all the hormonal treatments resulted in callus formation. In general, the highest callus volume was achieved in cotyledon explant treated with 3 mg^{-1} TDZ + 0.5 mg^{-1} IBA. Among different hormonal combinations tested in this study, BA/IBA combination was weaker than TDZ/IBA regarding callus formation. In all TDZ concentrations, the addition of IBA resulted in increased production and proliferation of callus. There was no significant difference among various concentrations. In the same way in an investigation conducted by Gomez-Lyva., *et al.* (2008) on *Arbutus unedo*, the highest callus mass was obtained by application of TDZ. Similar results were reported by

Shrma *et al.* (2011) on *Jatropha curcas*. Based on the results obtained in this study, it was revealed that both BA and TDZ were individually more effective in shoot formation; moreover, no significant difference was observed between the two hormones. Similarly, Lata *et al.* (2010) used calli obtained from leaf explant for shoot regeneration and observed the best regeneration rate in MS medium supplemented with 0.5 µM TDZ. Results reported by other authors suggest that high concentration of TDZ increases regeneration rate in some plants (19). It should be mentioned that the mechanism of action of this PGR has not been fully understood yet; however, there are two hypotheses in this regard. The first hypothesis expresses that the hormone induces regeneration by direct stimulation of tissue; while the second one state that TDZ stimulates indigenous cytokinins and thereby facilitates shoot regeneration (9). Besides genotype role, plant growth regulators and particularly auxins are involved in regeneration at plant tissue cultures (14). In some plant species, BAP hormone, especially when combined with an auxin such as NAA, significantly enhances somatic embryo formation (15). Individual evaluation of the effect of four concentrations of IBA and NAA on rooting in the regenerated shoots revealed that there was a significant difference among different IBA concentrations; however, the difference was not significant for NAA concentrations. Results showed that 0.1 mg^{-1} IBA had enormous effect on root formation so that by increase in IBA concentration, root formation rate was reduced. The roots formed under IBA treatment were longer and thinner than those formed under NAA treatments. Lata *et al.* (2010) applied IBA, IAA and NAA for root induction in the regenerated shoots and reported that ½MS supplemented with 2.5 µM IBA was the best medium for root formation. Alhadi *et al.*, (2011) found out that IBA is more effective than NAA in root induction.

The largest callus volume and highest callus fresh weight (3.15 gr) was obtained for cotyledon explant in MS medium supplemented with 3 mg^{-1} TDZ + 0.5 mg^{-1} IBA and MS medium contaninig 2 mg^{-1} TDZ + 0.5 mg^{-1} IBA, respectively. In shoot formation step, the highest rate of shoot regeneration was achieved in the calli produced from epicotyl explant treated with 2 mg^{-1} BA + 0.5 mg^{-1} IBA; and the highest length of regenerated shoots (1.23 cm) was observed in 2 mg^{-1} BA + 0.5 mg^{-1} IBA treatment. In general, cotyledon was the best explant and TDZ in combination with IBA was the best treatment for callus formation. Epicotyl explant also showed better regeneration compared to cotyledon. So, depending on the purpose (calli induction or indirect regeneration), both of explants could be used by culturing them on MS media containing TDZ in combination with IBA. The results obtained in this experiment paves the way for the application of biotechnology in breeding programs of this native and valuable medicinal plant.

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اثر ترکیبات مختلف TDZ و BA در باززایی این‌ویتروی شاهدانه ایرانی (*Cannabis Sativa*) با استفاده از ریزنمونه‌های کوتیلدون و اپی‌کوتیل

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چکیده

تحقیق حاضر به منظور بررسی امکان ریزازدیادی گیاه شاهدانه و تعیین محیط کشت بهینه و ترکیب مناسب تنظیم کننده‌های رشدی در شرایط این‌ویترو انجام شد. ریزنمونه‌های کوتیلدون و اپی‌کوتیل بدست آمده از گیاهچه‌های یک ماهه رشد یافته در محیط این‌ویترو بر روی محیط‌کشت MS حاوی (۰/۱، ۰/۲، ۰/۵، ۱، ۲ و ۳ میلی‌گرم در لیتر) هورمون BA و (۰/۱، ۰/۲، ۰/۵، ۱، ۲ و ۳ میلی‌گرم در لیتر) هورمون TDZ به تنهایی یا در ترکیب با ۰/۵ میلی‌گرم در لیتر IBA مورد استفاده قرار گرفتند. در بسیاری از تیمارهای تنظیم کننده‌های رشد مورد استفاده، تشکیل کالوس مقدم بر باززایی مستقیم بود. بین دو ریزنمونه مورد استفاده، ریزنمونه کوتیلدون دارای فراوانی بالاتر کالوس‌زایی بوده و بیشترین حجم کالوس در همین ریزنمونه در محیط کشت MS حاوی ۳ میلی گرم در لیتر همراه با ۰/۵ میلی‌گرم در لیتر IBA بدست آمد. در ریزنمونه کوتیلدون بیشترین وزن تر کالوس (۳/۱۵ گرم) در محیط کشت MS حاوی ۲ میلی‌گرم در لیترTDZ همراه با ۰/۵ میلی‌گرم در لیتر IBA بدست آمد. در مرحله ساقه‌زایی بیشترین میزان باززایی ساقه در کالوس‌های القاء شده از ریزنمونه اپی‌کوتیل تیمار شده با ۲ میلی‌گرم در لیتر BA همراه با ۰/۵ میلی‌گرم در لیتر IBA مشاهده شد و بیشترین طول ساقه‌های باززایی شده (۱/۲۴ سانتی‌متر) در تیمار ۲ میلی‌گرم در لیتر با ۰/۵ میلی‌گرم در لیتر IBA بدست آمد. در مجموع، ریزنمونه کوتیلدون ریزنمونه بهتر و تنظیم‌کننده رشد TDZ در ترکیب با IBA بهترین تیمار جهت کالوس‌زایی بود. ریزنمونه اپی‌کوتیل در مقایسه با کوتیلدون باززایی بهتری را نشان داد.

کلمات کلیدی: باززایی، تنظیم‌کننده‌های رشد، شاهدانه، کالوس.