IN VITRO REGENERATION RESPONSE OF OKRA (*ABELMOSCHUS ESCULENTUS* L.) UNDER THE EFFECT OF PHYTOHORMONES

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Abstract. *In vitro* multiplication of okra is known to be delicate due to its recalcitrant nature. The current study was conducted to investigate the potential applications of thidiazuron (TDZ) on okra's *in vitro* regeneration. Cotyledonary leaves, hypocotyls and apices were taken from 15-day old *in vitro* grown seedlings, they were then inoculated on Gamborg (B5) medium containing different plant growth regulators. Maximum callus formation (100%) was obtained from cotyledonary leaves and hypocotyls on B5 without hormones also by adding 0.5 mgL^{-1} TDZ and 1 mgL^{-1} BAP for cotyledonary leaves followed by B5 containing 1 mgL^{-1} TDZ and 1 mgL^{-1} BAP with 95.83% of calli formed from hypocotyls. B5 supplemented with 1 mgL^{-1} of TDZ gave the highest rate (100%) of direct regeneration from apices followed by Gamborg medium without PGRs and B5 containing an equimolar concentration of TDZ and BAP with 66.66% of shoot formation. This regeneration system can be used to produce healthy plantlets with a low risk of genetic instability and somaclonal variants.
Keywords: *okra, callogenesis, cotyledonary leaves, hypocotyls, apex*

Introduction

Okra is a vegetable crop of significance in the malvaceae family grown in tropical, subtropical and north-eastern African countries (Duzyaman and Vural, 2001; Irshad et al., 2017), presently distributed in a wider range from Asia through southern Europe to America. Its green fruit is commonly consumed as a vegetable due to its high protein content, fiber and mineral salts (Vietmyer, 2006; Gemede et al., 2015). In addition, okra seeds are rich in oil that contains unsaturated fatty acids such as linoleic acid which helps reduce LDL cholesterol (Dubey and Mishra, 2017) and its mucilage can be used as plasma replacement. It is also known for eliminating toxic substances in liver (Das et al., 2019), whereas fibers, that okra contains, help maintain balance and control sugar level in blood (Khosrozadeh et al., 2016; Durrazo et al., 2018).

In Algeria, okra is grown in the wilaya of Guelma and it is called a niche production since its culture is exclusive, low and traditional but it has increased worldwide throughout the years making India and Nigeria the largest producers of this crop (Varmudy, 2011; FAOSTAT, 2016) in terms of area, economic value and employment. It is confronted with a number of biotic constraints including various diseases (Appiah et al., 2020; Jamir et al., 2020) and abiotic stress like salinity (Achour et al., 2015; Yakoubi et al., 2019; Bensaida et al., 2019) which limit its production and degrade its quality (Dhankar et al., 2013; Kabir et al., 2016).

Therefore, great attention is attributed to varietal propagation of okra through biotechnological approaches (Roy and Mangot, 1989; Haider et al., 1993; Narendran et

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al., 2013; Rizwan et al., 2020) such as *in vitro* regeneration, also termed micropropagation, in order to increase stress/disease tolerance and to obtain plants with desirable agronomic traits (Irshad et al., 2017). The importance of this biotechnological tool lays in widening the possibilities for the development of somaclonal variants of different cultivars (Kabir et al., 2008).

The recalcitrant nature of okra hinders the attempts of its manipulation which leads to low regeneration frequencies (Anisuzzaman et al., 2010). Consequently, a wide range of media and different combination of growth regulators should be tested to establish effective protocols so as to optimize its callus induction and direct or indirect regeneration.

Explant type, culture media and the use of phytohormones are factors that affect *in vitro* propagation of many species like *Cicer arietinum* (Kadiri et al., 2014; *Atriplex halimus* (Halfaoui et al., 2018) and okra (Rizwan et al., 2020; Belkhodja and Belkhodja., 2022). Among plant growth regulators, thidiazuron (TDZ) is known as a potent synthetic growth regulator which can mimic the effects of both auxin- and cytokinins in plants. TDZ has been widely used to stimulate regeneration in various plant species ranging from herbaceous to tree species (Vinod, 2019). It also appears that it stimulates shoot bud by initiating cell proliferation in apical meristems and reprograming its differentiation (Vu et al., 2006; Dey et al., 2012). Furthermore, TDZ's impact on shoot proliferation is highly related to specific concentrations and species type (Ahmad and Faisal, 2018). The current study is conducted to better understand the role and investigate potential applications of TDZ for the induction of *in vitro* regeneration of okra.

Materials and methods

Plant material

Seeds of okra were harvested from northeastern Algeria, Nechmeya Wilaya of Guelma, *GPS coordinates* (Latitude: 36.6115, Longitude: 7.51331° 36′ 41″ North, 7° 30′ 48″ East), on a small area in 2020. Seeds were kept in cold (5°C) at the laboratory of Plant Physiology, Oran University.

Okra seeds were selected according to their size and shape (homogeneous sizes) to ensure the homogeneity of seedlings, plants used as a source to obtain explants.

Seed sterilization and in vitro germination

Seeds were first washed with tap water to get rid of impurities and dust. They were surface sterilized by imbibition in 30% laurylated mercryl solution for 10 min then treated with 8% sodium hypochlorite (NaClO) under laminar flow unit for 10 min. Seeds were rinsed 5 times with sterile distilled water to remove all residues then dried with sterile filter paper and distributed in glass jars (250 ml) at a rate of 6 seeds per jar on 0.7%water-agar (w:v), pH was adjusted to 5.8 before autoclaving at 120 °C for 20 min. The germination of seeds was at first conducted in a dark room at 26 °C, after 2 days they were transferred to growth chamber set at 27 °C with 16:8 h light/dark photoperiod.

Media preparation

The medium used in this experiment was a modified Gamborg (B5) medium, prepared from the macroelements of the Gamborg B5 medium (Gamborg et al., 1968) and the microelements and vitamins of MS medium (Murashige and Skoog, 1962),

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containing 30 g/l sucrose as a source of carbon and solidified with 8 g/l of agar. The medium was supplemented or not with plants growth regulators, 6-benzylaminopurine (BAP), Thidiazuron (TDZ) and 1-Naphthaleneacetic acid (NAA) at different concentrations (*Table 1*). The media were autoclaved at 120 °C for 20 min at a pressure of 2 bar after adjusting the pH to 5.8.

Table 1. *Different concentrations and combinations of PGRs (plant growth regulators) tested*

Culture media	Plant growth regulator (mgL^{-1})		
	TDZ	**BAP**	**NAA**
B5 (0)	0	0	0
B5 (1)	0.5	0	0
B5 (2)	1.0	0	0
B5 (3)	0.5	1.0	0
B5 (4)	1.0	1.0	0
B5 (5)	0	2.0	0.5
B5 (6)	1.0	0	0.5

Callus induction and regeneration

Explants of the cotyledonary leaves, hypocotyl and apex were taken from 15-day old in vitro grown seedlings, cut into small fragments about 5-6 mm long for the hypocotyls and 25 mm^2 area for the foliar explants, under strict aseptic conditions.

Explants, thus prepared, were inoculated at the rate of 6 explants per jar for hypocotyls, 4 explants per jar for cotyledonary leaves and 3 explants per jar for the apex in replica for each medium. The apex was placed so that the cauline meristem is perpendicular to the culture medium. The glass jars contained information on the date of inoculation, the explant type and the growing medium. They were then placed in a culture chamber that provides a 16-h photoperiod and a temperature of 26 ± 1 °C.

The macroscopic evolution of explants was monitored daily to note any changes. After one month of inoculation, the rate of callogenesis (*Eq. 1*) and micropropagation (*Eq. 2*) was expressed using the following equations:

$$\text{Callus induction frequency} = \frac{\text{Number of explants with callus}}{\text{Number of explants inoculated}} \text{ x100} \quad \text{(Eq.1)}$$

$$\text{Shoot induction frequency} = \frac{\text{Number of explants with shoot}}{\text{Number of explants inoculated}} \text{ x100} \quad \text{(Eq.2)}$$

Acclimatization

Plantlets with developed rooting were removed from B5 medium and rinsed with sterile distilled water, they were transferred into cups containing sterilized sand and compost at a ratio of 1:1 respectively. The plantlets were covered with transparent plastic bags to maintain moisture and placed in growth chamber at 26 ± 1 °C provided by cool white fluorescent tubes for 7 days. They were then transferred into pots containing regular soil and the plastic bags were removed for further growth, as described by Woldeyes et al. (2021) on okra.

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Statistical analysis

The various results obtained were the subject of a statistical study by an analysis of variance (ANOVA) to evaluate the effect of the factors on callogenesis and micropropagation, namely the type of explant and the phytohormones used. The difference between the average rates obtained was analyzed by the LSD tests, p-value ≤ 0.05 using STATISTICA 13.5.

Results

Callus induction

Monitoring the evolution of explants after the culture reveals a precocity and a variability of the callogenic power. Hypocotyls react first (after the sixth day), with swelling sometimes accompanied by tearing of the epidermis under the effect of internal cellular activity (*Fig. 2a*). They were then followed by the cotyledonary leaves which begin to show morphological changes such as winding, turgescence and then the formation of callus observed at the level of the excised zones. These calli vary in color from white, light green, beige to light brown (*Fig. 2b*). Browning of tissues and necrosis are also observed in all types of explants.

It was observed that 100% callus induction was achieved on B5 media without hormones for both explants. Adding TDZ solely at 1 mgL^{-1}, allowed 70.83% of hypocotyls and 91.66% of cotyledonary leaves to express their callogenic potential whereas at 0.5 mgL^{-1}, 0% of callusing was reported. Combination of TDZ with BAP was favorable for callusing with a rate of 100% for cotyledonary leaves explants on B5 supplemented with 0.5 mgL^{-1} TDZ and 1 mgL^{-1} BAP and 95.83% of calli formed from hypocotyls on B5 containing 1 mgL^{-1} TDZ and 1 mgL^{-1} BAP. However, the use of NAA either with TDZ or BAP did not improve callus induction (*Fig. 1*).

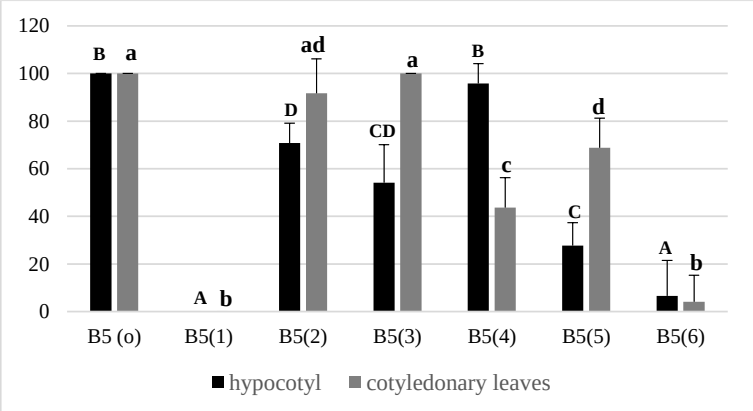

Figure 1. *Callus induction frequency in response to PGR treatment. TDZ: BAP: NAA mgL^{-1}; B5 (0) (0:0:0) B5 (1) (0.5:0:0) B5 (2) (1:0:0) B5 (3) (0.5:1:0) B5 (4) (1:1:0). B5 (5) (0:2:0.5) B5 (6) (1:0:0.5). Values are means ± standard error; data were recorded after four weeks of culture, means with the same letters are not significantly different regarding the same explant, with LSD test and p-value ≤ 0.05*

The evaluation of these results by statistical tests (*Table 2*) reveals that callus induction in okra is influenced by the type of explant and the concentration of exogenous plant

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growth regulators. The analysis of variance showed that the two factors studied have a highly significant effect on the induction of callogenesis as well as their interactive effects.

Table 2. *F-value and P-value obtained from ANOVA showing the effect of explant type and the combinations used in media on callogenesis of okra*

	ddl	MC	F	P
Explant	1	728.0	6.875	0.012401*
Media	6	11207.5	105.843	0.000000**
Explant × media	6	2115.9	19.982	0.000000**

**Highly significant $p < 0.01$. *Significant $p < 0.05$

Regeneration of okra and acclimatization

After statistical analysis, ANOVA revealed a highly significant effect of concentrations and combinations of PGRs on the micropropagation of okra and shoot length (*Table 3*). Morphogenetic responses of apex explants are presented (*Fig. 2c, d*). Our results showed that direct *in vitro* regeneration yielded plantlets with different growth rates depending on culture media (*Table 4*).

Table 3. *Effect of concentrations and combinations of PGRs on the micropropagation of okra and shoot length*

	ddl	MC	F	P
PGRs on micropropagation	6	4452.11	27.5475	0.000000**
PGRs on shoot length	6	24.752	66.435	0.000000**

**Highly significant $p < 0.01$

Among the tested media, B5 supplemented with 1 mgL^{-1} of TDZ gave the highest rate (100%) followed by both Gamborg medium B5 (0) without PGRs and B5 (4) containing an equimolar concentration of TDZ and BAP (1/1 mgL^{-1}) with 66.66% of regeneration, TDZ's concentration increase either used alone or combined with BAP seems to be effective for shoot induction of okra. The combination of NAA with TDZ and NAA with BAP did not enhance the micropropagation of apices therefore low frequencies were registered with 33.33% and 16.66% respectively.

Length measurements were taken from induced shoots after 4 weeks of culture on media containing different combinations and concentrations of PGRs (*Table 4*). The combination of TDZ with BAP (1/1 mgL^{-1}) increases shoot length with a mean length of 10.28 ± 0.94 cm followed by B5(0) without hormones with 7.36 ± 0.86 cm. On the other hand, media containing 0.5 mgL^{-1} NAA + 2 mgL^{-1} BAP and 0.5 mgL^{-1} NAA + 1 mgL^{-1} TDZ did not show a significant effect on plant height with maximum shoot length of 4.34 ± 0.46 cm and 4.46 ± 0.43 cm respectively. Among the tested media, TDZ at 0.5 mgL^{-1} alone or combined with BAP, was not effective for shoot length.

Well rooted *in vitro* developed plantlets were rinsed with sterile distilled water (*Fig. 2e*) then transferred to sterilized sand and compost mixture. The plantlets were covered with transparent plastic bags to maintain humidity placed in growth chamber for 7 days. They were then transferred into pots containing regular soil and the plastic bags were removed for further growth (*Fig. 2f*).

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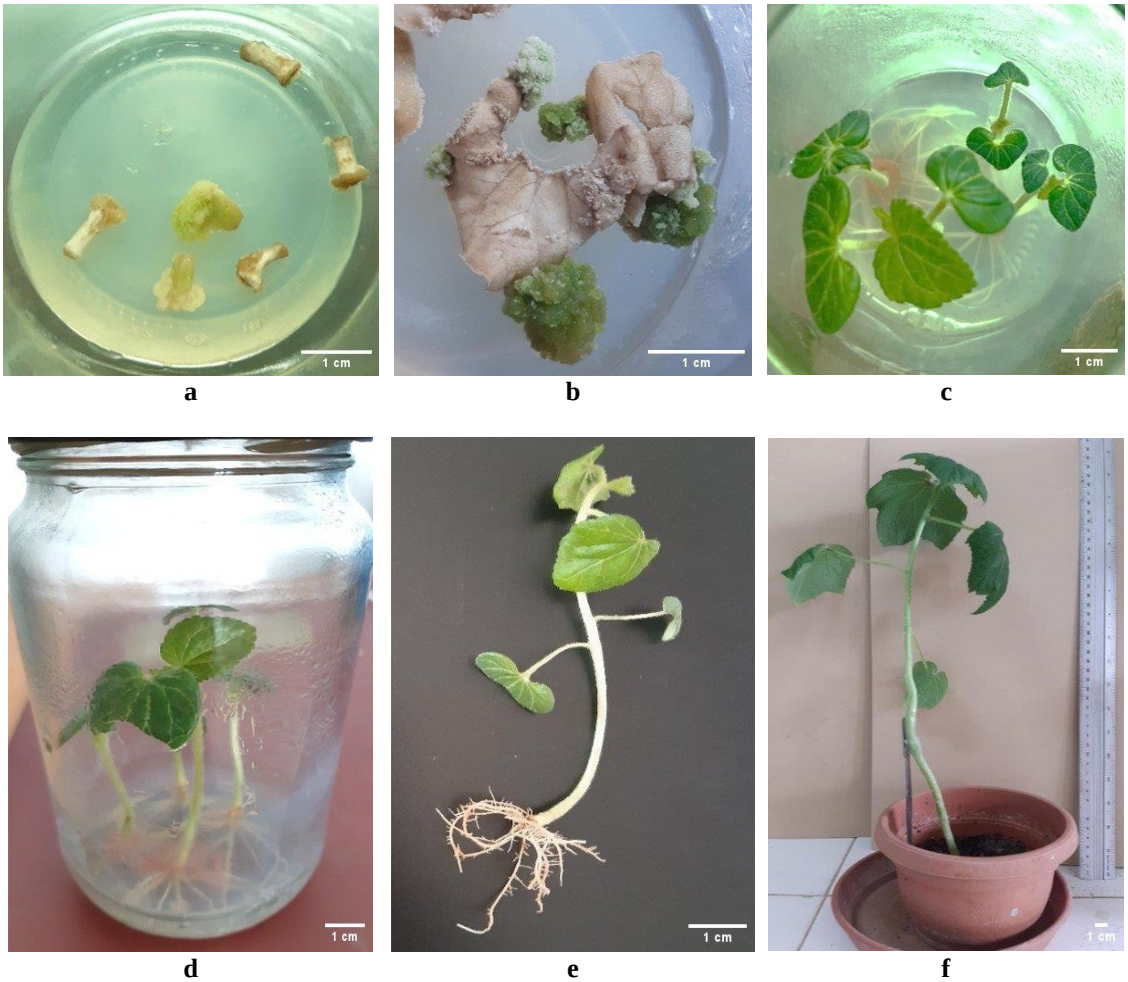
Table 4. *Effect of different concentrations and combinations of plant growth regulators on shoot induction from apex explant of okra*

Culture media	Plant growth regulators (mgL^{-1})			Regeneration frequency (%)	Shoot length (cm)
	TDZ	BAP	NAA		
B5(0)	0	0	0	66.66 ± 0.00 ^{a}	7.36 ± 0.86 ^{d}
B5(1)	0.5	0	0	49.99 ± 19.24 ^{ad}	5.08 ± 0.13 ^{b}
B5(2)	1.0	0	0	100.00 ± 0.00 ^{e}	6.42 ± 0.44 ^{c}
B5(3)	0.5	1.0	0	13.33 ± 18.25 ^{b}	4.16 ± 0.57 ^{a}
B5(4)	1.0	1.0	0	66.66 ± 0.00 ^{a}	10.28 ± 0.94 ^{e}
B5(5)	0	2.0	0.5	16.66 ± 19.24 ^{bc}	4.34 ± 0.46 ^{ab}
B5(6)	1.0	0	0.5	33.33 ± 0.00 ^{cd}	4.46 ± 0.43 ^{ab}

Values are means ± standard error; data were recorded after four weeks of culture, means followed by the same letter within a column are not significantly different according to LSD test with p-value ≤ 0.05



a **b** **c**

d **e** **f**

Figure 2. *Callus formation and direct in vitro regeneration of okra under the influence of PGRs. (a) Callus formation from hypocotyl. (b) Induced calli from cotyledonary leaves. (c) Shoot induction on B5 + 1 mgL^{-1} TDZ. (d) Shoot induction on B5 + 1 mgL^{-1} TDZ and 1 mgL^{-1} BAP. (e) In vitro regenerated plants showing rooting on B5 without PGRs. (f) Acclimatization of plantlet in soil*

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Discussion

Callogenesis

This work was conducted to study and optimize conditions for callogenesis and organogenesis of okra.

The results showed that callogenesis was always preceded by an increase in the volume of the explants. These are made up of differentiated tissues and their *in vitro* culture provoke a reprogramming of specific target cells (Ikeuchi et al., 2018) in response to plant growth regulators and the medium's nutritive elements; an acquisition of a mitotic activity which initially leads to the formation of an undifferentiated cellular cluster called "callus" (Margara, 1984).

Previous studies showed that callogenesis is highly affected by the concentration and the combination of plant growth regulators added to the media. According to our findings, high callus induction was noted on B5 medium supplemented with 0.5 or 1 mgL^{-1} TDZ and 1 mgL^{-1} BAP combinations. The calli were green to yellowish in color and compact in nature for the explants of hypocotyls and white and friable for cotyledonary leaves this result is in accordance with the works of Kabir et al. (2008) on okra, contrary to the work of Sharma and Shahzed (2008) on *Abelmoschus moschatus* Medik. L when MS was supplemented with TDZ and NAA.

According to Murthy (1998) nodular and green calli are initiated from geranium leaf explants when TDZ is used at low concentrations or combined with BAP; this is consistent with the findings of this experiment where high callogenesis rates were observed in medium supplemented with TDZ and BAP.

In addition, Guo et al. (2011) find that TDZ exhibits effects similar to auxins and cytokinins, although it differs in chemical structure. According to the same authors, they also add that TDZ can modify the functioning of endogenous growth regulators, directly or indirectly, and produce reactions in the cells and their membranes necessary for their regeneration by improving the supply of purines and inhibiting the functioning of cytokinin oxidases responsible for their degradation (Casanova et al., 2004). TDZ also induces the activation of adenines to adenosines which results in a high energy level necessary for cell multiplication and/or organogenesis of explants (Murthy et al., 1998; Verma et al., 2016). Wang et al. (1991) reported that TDZ-stimulated enzymes are associated with the cell wall and cell membrane including modification of its fluidity.

In our study, the highest callogenesis rate was observed in B5 medium without any growth regulator, however calli formed seemed generally heterogeneous, this probably due to the culture conditions, mineral composition of the medium or the endogenous phytohormones and the totipotence of plant cells and explants anatomy (Michel et al., 2008; Gueye et al., 2009). According to Choi et al. (2003), callus exogenous hormones independent formation can be achieved with low salt strength from Cotyledon explants of *Panax ginseng*.

In this study, the combination of TDZ with an auxin (NAA) did not seem favorable for callus formation since it presented a low rate of callogenesis and the hypocotyl and cotyledonary leaves did not respond to tissue culture, our findings are in accordance with the work of Rizwan et al. (2020) on okra where the lowest callus induction frequency was observed on a medium supplemented with 0.5 mgL^{-1} NAA + 0.5 mgL^{-1} TDZ from cotyledon and hypocotyl explants. Rizwan et al. (2020) also reported that hypocotyl explants presented higher callogenesis rate in comparison to other explants, so our study is in agreement with these findings.

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Plant regeneration

Direct in vitro regeneration is significantly influenced by internal and external factors namely type of growth regulators, concentrations and combinations used. The purpose of this study was to examine and understand the role of TDZ on shoot induction in apex explants isolated from 15-day old seedlings of *Abelmoschus. esculentus* L. Direct in vitro regeneration was obtained on B5 medium without hormones and with TDZ alone or combined with BAP. To date, no or very limited studies have been conducted regarding the use the B5 medium on okra, our methodology allowed encouraging and effective results on B5 (0), no PGR added, with 66.66% of regeneration frequency, these results are not to be neglected and may be due to genetic structure of explant (Rizwan et al., 2018) and medium texture and composition (Anisuzzaman et al., 2010) and probably to the endogeneous richness of hormones, that is already reported by Belkhodja and Belkhodja (2022) on the same species.

The application of 0.5 mgL^{-1} TDZ alone on B5 medium appeared to be significantly advantageous for shoot induction, this is consistent with the work of Sharma and Shahzed (2008) who reported that TDZ was proved critical for multiple shoot induction from cotyledonary node explant of a malvaceae *Abelmoschus moschatus* Medik. L. The increase of concentration of TDZ (1 mgL^{-1}) gave the highest rate of regeneration (100%), an explanation for improved regeneration response may lie in TDZ's unique property of having stimulatory effect on the accumulation of cytokinins by preventing their breakdown to adenine and adenosine (Hutchinson et al., 1996). Cytokinins have been demonstrated to have an important function at regulating cell multiplication and elongation and controlling shoot development and proliferation (Mok and Mok, 2001); BAP is plant regulator that belongs to cytokinin group which has the potential for micropropagation induction (Ashraf et al., 2014). Unlike BAP, TDZ is resistant to cytokinin disintegrating enzymes (Dey et al., 2012). supplementing B5 medium with 1 mgL^{-1} of TDZ and 1 mgL^{-1} of BAP showed high precent of direct shoot regeneration. Our result matches with the earlier reports on the same species (Rizwan et al., 2018) and on *Oryza sativa* L. (Noor et al., 2022).

The association of TDZ and BAP gave plantlets that marked the highest average lengths with 10.24 ± 0.94 cm and good rhizogenesis was reported, followed by B5 medium without growth hormones with an average length value of 7.28 ± 0.86 cm along with normal root production. In contrast, Anissuzaman et al. (2008) reported that rooting formation occurred only on MS medium supplemented with NAA and IAA. Furthermore, necrosis of plantlets, characterized by browning of tissue, was observed along with poor rooting on B5 supplemented with 2 mgL^{-1} BAP and 0.5 mgL^{-1} NAA, according to Rizwan et al. (2018) this browning is due to the secretion of phenolic compounds that results an eventual death of small explants.

Conclusion

This study allowed direct in vitro regeneration of okra from apex explants on Gamborg media supplemented with plant growth regulators. Maximum callus formation was obtained on B5 medium with no hormones for both explants; the combination of TDZ with BAP appeared to be effective for callusing. Shoot induction was highly influenced by plant growth regulators where TDZ at 1 mgL^{-1} proved to be the optimum concentration and the most suitable for micropropagation of Okra. This regeneration system can be used to produce healthy plantlets with a low risk of genetic instability and somaclonal variants.

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