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Research Report

In vitro Regeneration of *Abelmoschus esculentus* L. cv. Wufu: Influence of Anti-browning Additives on Phenolic Secretion and Callus Formation Frequency in Explants

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Abstract. Okra (*Abelmoschus esculentus* L.) belongs to the Malvaceae family and is a difficult species to manipulate and regenerate in vitro. In this study, factors that influence secretion of phenolic compounds, callus induction, and subsequent regeneration in okra were optimized. Different concentrations of anti-browning additives, such as activated charcoal (AC) and citric acid (CA) were tested alone or in combination with ascorbic acid (AA) in MS medium to evaluate their ability to control phenolic secretion from hypocotyl and cotyledon explants. Among the anti-browning additives, 200 $mg \cdot L^{-1}$ AC and 10 $mg \cdot L^{-1}$ CA + AA (1:1) were best at controlling phenolic secretion from hypocotyl and cotyledon explants in callus induction medium. Hypocotyl explants were more suitable for callus initiation and organogenesis than cotyledon explants. Moreover, among twelve types of media tested for callus culture, the addition of 0.5 $mg \cdot L^{-1}$ 2,4-dicholorophenoxy acetic acid (2,4-D) and 1.5 $mg \cdot L^{-1}$ benzyladenine (BA) to the growth media was the most effective for hypocotyl explants, whereas MS + 1.5 $mg \cdot L^{-1}$ α-naphthylacetic acid (NAA) and 0.5 $mg \cdot L^{-1}$ 6-benzyloaminopurine (BAP) was effective for cotyledon explants. Improved shoot regeneration was achieved with 2 $mg \cdot L^{-1}$ BAP and 0.1 $mg \cdot L^{-1}$ indole butyric acid (IBA). The maximum number of strong and healthy roots was obtained on media that included 2 $mg \cdot L^{-1}$ IBA and 200 $mg \cdot L^{-1}$ AC. Rooted plantlets were successfully acclimatized in earthen pots, and the rooted plantlets had normal morphology and growth characteristics. The present study offers a potential solution for controlling phenolic secretion during okra regeneration using additives and evaluates the manipulation of plant growth regulators for effective regeneration of the Wufu okra cultivar.

***Additional key words*:** adsorbent, antioxidants, browning, okra, plant growth regulators, tissue culture

Introduction

Okra (*Abelmoschus esculentus* L.) is an economically important vegetable crop grown in tropical, subtropical, and Mediterranean countries (Düzyaman and Vural, 2001). In Asia, the fruit of *A. esculentus* is most commonly used as a vegetable (Tripathi et al., 2011). Okra, despite being a minor crop, has gained considerable interest as a traditional vegetable throughout the world due to its share of vitamins, calcium, and mineral salts for the human diet (Gemede et al., 2015). In addition, because of its high fiber and balanced seed protein content, okra is known as a "perfect villager's vegetable" in Africa (Vietmyer, 2006). Okra is susceptible to a number of biotic and abiotic stresses that greatly impair its production and degrade its quality (Dhankar et al., 2013).

Classical and mutation breeding programs have rarely succeeded in developing okra varieties that are resistant to biotic and a biotic stresses (Narendran et al., 2013; Rajamony et al., 2004). Genetic engineering has become a widely used method to increase stress tolerance, improve nutrient efficiency and enhance productivity of food crops. The development of transgenic plants is primarily based on efficient in vitro regeneration systems; therefore, in vitro culture of okra may be an alternate method for developing okra plants with desirable agronomic traits. In vitro tissue culture and regeneration of okra from nodes and shoot tips was reported more than 30 years ago (Mangat and Roy, 1986). Since then, several attempts have been made to efficiently regenerate different okra cultivars using different explants sources (Anisuzzaman et al., 2010; Kabir et al., 2008; Narendran et al., 2013). However, the recalcitrant nature of okra hinders its in vitro manipulation and adversely affects its regeneration

frequency (Anisuzzaman et al., 2010; Narendran et al., 2013). Explant type, plant growth regulators, culture condition, phenolic compound secretion, and the browning of callus tissues are factors that affect callus culture and subsequent in vitro regeneration of okra. Among these factors, phenolic compound secretion from explants into the culture media and callus browning are well-known problems that adversely affect okra regeneration (Anisuzzaman et al., 2010). Phenolic secretion is caused by injuries during the isolation of explants and the oxidation of these phenolic compounds on cut surfaces can cause necrosis, which hinders nutrient uptake and results in the death of explants (Erland and Mahmoud, 2014; Yildirim and Turker, 2014). Polyphenol oxidase (PPO) enzyme is thought to play a key role in the oxidation of phenolic compounds (Larson, 1988); other oxidative enzymes, such as phenylalanine ammonia lyase (PAL) and peroxidase (POD) also affect the oxidation of phenolic substances at cut surfaces of explants (Andersone and Ievinsh, 2002; Tabiyeh et al., 2005).

To reduce the effects of PPO and POD on phenolic compound oxidation and the subsequent effects of oxidized phenolic compounds on tissue culture, a number of culture manipulations have been developed. These include the initial culture of explants in darkness (Da Silva et al., 2015) or under low temperature (Nguyen et al., 2007; Rather et al., 2011), pretreatment of explants with antioxidants (Ahmad et al., 2016), and the culture of explants on medium supplemented with adsorbents and antioxidants (Klenotičová et al., 2013; Meziani et al., 2016). In earlier studies, whether the addition of an adsorbing additive (activated charcoal) and antioxidants (citric acid and ascorbic acid) to the culture medium was either beneficial or not depended on the species or type of explant that was being propagated (Jafarkhani Kermani et al., 2008; Pankaj et al., 2014). In this study, we investigated the role of adsorbing agents and antioxidants to control tissue browning and cell proliferation in explants in an okra tissue culture system. The knowledge gained from these experiments could benefit future breeding and transformation of okra crops. To our knowledge, this is the first demonstration of callus induction in okra using anti-browning additives in the tissue culture medium. In addition, we present a protocol for the in vitro regeneration of the Wufu okra cultivar using an adsorbent and antioxidants in the culture medium to suppress phenolic secretion from cotyledon and hypocotyl explants.

Materials and Methods

Seed Material

Seeds of *A. esculentus* cultivar Wufu were collected from the germplasm bank of the Institute of Vegetable Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian, China. Seeds were washed in double distilled water (ddH_2O) (×5) and then surface-sterilized with 0.1% (w/v) $HgCl_2$ for three minutes. Residual $HgCl_2$ was removed by washing the seeds three times in ddH_2O. Thereafter, seeds were treated with 70% ethanol for 30 s followed by an additional wash with ddH_2O (×3). Seeds were imbibed overnight in ddH_2O and then cultured in sterilized 200 ml baby food jars (5 seeds per jar) on half-strength Murashige and Skoog (MS) basal solid medium (Murashige and Skoog 1962) without plant growth regulators (PGRs). The sterilization and culturing processes were carried out in a sterilized environment in a laminar flow unit. For all experiments, 0.8% agar (w/v) and 2.5% sucrose (w/v) were added to the MS medium, and the pH was adjusted to 5.8 with 0.5 N NaOH or HCl before autoclaving solutions at 121°C for 20 minutes. For CA and AA treatments, the pH of CA and AA solutions was adjusted to 5.8 in 50 ml of MS basal medium, passed through a 0.22-μm filter, and then added to autoclaved media. Cultured seeds were incubated for 3 days in the dark in a culture room at 25 ± 2°C and 70% relative humidity, after which the seeds were transferred to a culture room with a 16:8 h light/dark photoperiod. The light was supplied by cool white fluorescent lamps (Philips, Beijing, China) at an intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All chemicals were purchased from Macklin Co. Ltd. (Shanghai, China)

Media Fortification with Anti-browning Additives

The anti-browning additives AC, CA, and CA + AA, were tested at different concentrations (10-400 $mg \cdot L^{-1}$) to investigate their effects on controlling phenolic compound secretion and callus induction efficiency (Table 1). Hypocotyl and cotyledon explants were cultured on MS medium supplemented with different concentrations of anti-browning additives. The results were evaluated after two weeks of culture, and the best anti-browning additives were used in further experiments.

Callus Induction

Hypocotyl and cotyledon explants were aseptically excised from 11-day-old in vitro-grown seedlings. Hypocotyl explants were cut into 5-7 mm-long pieces, and cotyledons were cut into 5 mm small squares. For callus initiation, explants were inoculated in Petri-plates (15 × 90 mm) containing 30 ml of MS medium supplemented with PGRs and anti-browning additives (Table 1). Cultures were maintained in a culture room with a 16:8 light/dark photoperiod (40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 25±2°C. After 2 weeks of incubation, the percentage of phenolic compound secretion and callus induction frequency were determined (Fig. 2a and 2b, Table 2-4).

Table 1. Concentrations and combinations of PGR supplements and anti-browning additives tested in callus response and phenolic secretion control from cotyledon and hypocotyl explants

Treatment	PGRs mg·L^{-1}					Treatment	Anti-browning additives mg·L^{-1}		
	NAA	*BAP*	*2,4-D*	*BA*	*T-DZ*		*AC*	*CA*	*AC*
M1	1	0.5	-	-	-	A1	100	-	-
M2	1	1	-	-	-	A2	200	-	-
M3	1.5	0.5	-	-	-	A3	300	-	-
M4	2	0.5	-	-	-	A4	400		-
M5	-	-	0.5	0.5	-	A5	-	10	-
M6	-	-	0.5	1	-	A6	-	20	-
M7	-	-	1	1		A7	-	30	-
M8	-	-	0.5	1.5	-	A8	-	40	-
M9	-	-	0.5	-	0.5	A9	-	5	5
M10	-	-	0.5	-	1	A10	-	10	10
M11	-	-	1	-	1	A11	-	15	15
M12	-	-	1	-	1.5	A12	-	20	20

The labels M1-M12 represent PGRs combination only, A1-A12 represent anti-browning additives only, PGRs Plant growth regulators, NAA- α-naphthylacetic acid, BAP- 6-benzyloaminopurine, 2,4-D- 2, 4-Dicholorophenoxy acetic acid, BA- Benzyladenine, TDZ- Thidiazuron, AC- Activated Charcoal, CA-citric Acid, AC - Ascorbic Acid.

Table 2. Effect of NAA and BAP combinations with anti-browning additives on callus induction from cotyledon and hypocotyl explants

Anti-browning additives mg·L^{-1}	NAA + BAP mg·L^{-1}							
	0.5 + 0.5		1 + 1		1.5 + 0.5		2 + 0.5	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Control	7.33±0.88^{cde}	4.67±1.45^{ef}	5.00±0.58^{def}	4.00±0.58^{e}	9.33±0.88^{cde}	6.67±1.45^{fg}	5.00±1.15^{cdef}	5.00±0.58^{f}
AC								
100	7.00±1.53^{cde}	20.00±2.08^{a}	10.00±1.15^{abcde}	18.00±2.08^{ab}	13.67±0.88^{cd}	**25.00±1.73^{ab}**	11.00±1.53^{abcd}	17.67±1.45^{abc}
200	7.00±1.53^{cde}	17.67±1.45^{ab}	11.67±1.67^{abcd}	15.00±1.53^{bcd}	**14.67±1.45^{cd}**	22.00±1.15^{bc}	8.67±0.67^{bcde}	12.00±1.15^{cde}
300	3.00±0.58^{de}	6.67±0.88^{def}	4.00±0.58^{ef}	10.00±1.15^{cde}	8.00±0.58^{de}	6.67±0.33^{fg}	3.33±0.88^{ef}	10.00±1.73^{def}
400	0.00±0.00^{e}	2.67±0.33^{f}	0.00±0.00^{f}	6.00±1.00^{e}	0.00±0.00^{e}	2.67±0.33^{g}	0.00±0.00^{f}	6.33±1.76^{ef}
CA								
10	17.33±1.45^{ab}	18.33±2.03^{ab}	13.00±0.58^{abcd}	23.00±2.08^{a}	**25.00±1.73^{ab}**	**30.00±2.08^{a}**	12.67±1.76^{abc}	21.00±1.00^{a}
20	17.00±1.15^{ab}	15.00±1.73^{abc}	11.00±1.53^{abcd}	16.67±0.88^{abc}	18.00±1.73^{bc}	20.00±1.15^{bcd}	5.00±2.08^{def}	16.00±1.73^{abcd}
30	10.67±1.86^{bc}	11.67±1.45^{bcd}	10.33±0.88^{abcde}	8.33±0.88^{de}	15.00±1.73^{cd}	14.33±1.20^{de}	4.67±0.33^{def}	12.00±1.73^{cde}
40	6.67±1.86^{cde}	7.33±1.20^{def}	6.33±1.33^{cdef}	5.00±0.58^{e}	11.00±2.08^{cd}	10.00±1.53^{ef}	0.67±0.67^{f}	6.67±0.88^{bcde}
CA +AC								
5+5	20.00±1.53^{a}	17.33±1.20^{ab}	14.67±1.45^{abab}	23.00±2.52^{a}	**30.00±3.61^{a}**	18.67±0.88^{bcd}	17.00±1.53^{a}	19.00±1.15^{ab}
10+10	9.00±1.53^{cd}	13.67±1.76^{abcd}	16.33±1.76^{a}	**24.33±2.85^{a}**	17.67±2.40^{bc}	19.00±2.08^{bcd}	14.33±1.76^{ab}	15.67±1.76^{abcd}
15+15	7.00±1.15^{cde}	9.33±1.45^{cdef}	8.67±2.03^{bcde}	9.33±0.88^{cde}	10.33±1.45^{cd}	21.00±1.15^{bcd}	5.33±0.88^{cdef}	16.67±1.45^{abc}
20+20	3.00±1.00^{de}	3.50±1.50^{ef}	5.00±2.08^{def}	5.33±0.88^{e}	6.33±1.76^{de}	16.33±1.45^{cde}	1.33±0.88^{f}	12.67±1.45^{bcde}

AC-activated charcoal, CA-citric acid, AA-ascorbic acid. Data were collected after two weeks of culture on MS medium fortified with different concentrations of NAA + BAP along with anti-browning additives; values are means ± standard error (SE) from three replicated experiments, and those followed by a different letter within a column are significantly different at $p < 0.05$ according to Turkey's test. Best results are shown in bold.

Callus Growth and Development

Healthy and good-looking calli were transferred to baby jars containing 40 ml of MS medium fortified with different concentrations and combinations of auxins (NAA and 2,4-D) and the cytokinins thidiazuron (TDZ), BA, and BAP (Solarbio, Beijing, China) for callus development and growth. Subsequent culturing was performed after every two weeks using the medium that produced the best results.

Shoot Induction and Elongation

Well-developed calli with a granular structure were inoculated in MS medium supplemented with various plant growth regulators (Table 5) for shoot induction. The shoot regeneration percentage and the mean number of shoots per callus clump were recorded weekly for 60 days. The regenerated shoots were subcultured 2-3 times on the same medium to obtain the desired length.

Root Formation and Acclimatization of Plantlets

Elongated shoots approximately 3-4 cm in length were transferred to root induction medium (Table 6). Upon successful rooting, plantlets were transferred to small pots containing

Table 3. Effect of 2,4-D and BA in combination with anti-browning additives on callus induction from cotyledon and hypocotyl explants

Anti-browning additives mg·L^{-1}	2,4-D + BA mg·L^{-1}							
	0.5 + 0.5		0.5 + 1		1 + 1		0.5 + 1.5	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Control	5.67±0.88^{def}	4.33±0.88^{e}	5.33±0.88^{de}	4.67±0.88^{f}	7.00±0.58^{bc}	5.00±1.73^{e}	6.00±1.53^{cdefg}	5.33±0.88^{f}
AC								
100	10.67±0.67^{bcd}	17.33±2.33^{bc}	8.33±0.88^{cd}	30.00±3.51^{ab}	10.00±1.15^{ab}	17.00±1.53^{abc}	**11.33±1.86^{abc}**	31.67±2.40^{b}
200	6.00±0.58^{def}	30.00±1.73^{a}	9.33±1.45^{bcd}	35.00±3.21^{a}	4.67±1.20^{bcd}	21.00±2.31^{a}	7.00±1.15^{cdef}	**67.67±3.48^{a}**
300	3.67±0.88^{ef}	12.33±2.60^{bcde}	4.00±1.53^{de}	20.00±2.08^{bc}	3.00±1.00^{cd}	10.00±1.73^{bcde}	3.67±0.88^{efg}	19.00±2.08^{cde}
400	0.00±0.00^{f}	8.33±2.03^{cde}	0.00±0.00^{e}	16.33±1.45^{cde}	0.00±0.00^{d}	6.00±2.08^{de}	0.00±0.00^{g}	15.00±1.53^{def}
CA								
10	**16.33±2.33^{ab}**	19.00±1.15^{bc}	10.67±1.76^{bcd}	**23.33±2.33^{bc}**	14.67±2.19^{a}	18.33±2.40^{ab}	14.00±1.15^{ab}	18.33±2.40^{cde}
20	13.67±0.88^{bc}	21.00±2.08^{ab}	15.67±2.33^{ab}	16.67±1.86^{cde}	6.33±1.20^{bc}	18.00±2.08^{ab}	11.00±1.15^{abcd}	19.00±2.65^{cde}
30	11.67±1.76^{bcd}	18.67±3.18^{bc}	8.33±0.88^{cd}	12.33±2.03^{cdef}	7.67±1.45^{bc}	16.00±1.15^{abc}	10.00±1.15^{bcde}	16.00±0.58^{cdef}
40	7.67±1.67^{cde}	14.67±2.60^{bcde}	4.33±0.88^{de}	8.33±2.60^{def}	3.67±1.45^{cd}	12.00±1.53^{abcde}	5.67±1.33^{cdefg}	11.67±1.33^{def}
CA +AC								
5+5	**22.00±1.15^{a}**	17.33±1.45^{bc}	19.33±1.45^{a}	18.00±1.53^{cd}	15.00±1.15^{a}	20.00±1.53^{a}	16.67±2.03^{a}	26.67±2.73^{bc}
10+10	11.33±1.45^{bcd}	16.67±1.76^{bcd}	13.00±1.53^{abc}	17.33±1.86^{cde}	6.67±1.20^{bc}	16.00±2.65^{abc}	4.67±1.20^{defg}	22.33±3.18^{bcd}
15+15	9.67±1.76^{bcde}	10.67±1.76^{bcde}	9.67±1.76^{bcd}	8.67±1.45^{def}	3.00±0.58^{cd}	8.33±0.88^{cde}	4.00±0.58^{efg}	13.33±1.86^{def}
20+20	5.67±1.76^{def}	6.33±1.45^{de}	5.67±1.76^{de}	4.50±2.50^{f}	0.33±0.33^{d}	5.50±0.50^{e}	1.00±0.58^{fg}	10.00±2.00^{ef}

AC-activated charcoal, CA-citric acid, AA-ascorbic acid. Data were collected after two weeks of culture on MS medium fortified with different concentrations of 2,4-D + BA along with anti-browning additives; values are means ± standard error (SE) from three replicated experiments, and those followed by a different letter within a column are significantly different at $p < 0.05$ according to Turkey's test. Best results are showed in bold.

Table 4. Effect of 2,4-D and TDZ in combination with anti-browning additives on callus induction from okra cotyledon and hypocotyl explants

Anti-browning additives mg·L^{-1}	2,4-D + TDZ mg·L^{-1}							
	0.5 + 0.5		0.5 + 1		1 + 1		0.5 + 1.5	
	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl	Cotyledon	Hypocotyl
Control	5.67±0.88^{cde}	4.67±1.20^{fg}	8.33±2.19^{bcde}	4.00±0.58^{f}	6.00±0.58^{bcde}	4.33±1.33^{de}	8.00±0.58^{bcde}	4.00±0.58ef
AC								
100	8.33±0.88^{bcd}	24.00±2.08^{ab}	9.67±0.88^{bcde}	27.33±2.96^{ab}	5.67±0.88^{cde}	20.00±1.73^{b}	**9.67±1.45^{bcde}**	27.33±2.60^{ab}
200	4.67±0.33^{de}	29.67±1.76^{a}	8.33±0.88^{bcde}	34.67±2.85^{a}	4.00±0.58^{cde}	29.33±2.33^{a}	6.33±1.76^{cdef}	**36.00±2.08^{a}**
300	3.00±1.00^{ef}	13.33±2.03^{cdef}	2.67±0.67^{ef}	18.67±2.03^{bcd}	2.67±1.76^{de}	20.00±2.08^{b}	5.00±1.15^{def}	19.67±1.45^{bc}
400	0.00±0.00^{f}	9.33±1.76^{efg}	0.00±0.00^{f}	14.67±2.60^{cde}	0.00±0.00^{e}	16.00±2.52^{bc}	0.00±0.00^{f}	16.00±1.53^{c}
CA								
10	11.00±0.58^{ab}	20.33±1.45^{bcd}	**13.67±1.76^{abc}**	20.00±2.08^{bc}	10.00±1.15^{bc}	12.33±1.45^{bc}	13.00±1.15^{abc}	16.67±2.03^{c}
20	10.00±1.15^{ab}	**21.67±0.88^{abc}**	13.67±0.88^{abc}	17.67±1.45^{bcd}	9.33±0.88^{bcd}	16.33±2.33^{bc}	11.00±1.15^{bcd}	18.33±2.40^{bc}
30	8.33±0.88^{bcd}	16.33±0.88^{bcde}	10.67±1.20^{abcd}	18.00±1.53^{bcd}	9.33±1.86^{bcd}	14.67±0.88^{bc}	11.33±1.20^{abcd}	16.67±0.88^{c}
40	4.33±0.33^{de}	12.33±0.88^{def}	6.33±1.33^{cdef}	14.33±1.20^{cde}	5.33±2.33^{cde}	10.67±0.88^{cd}	7.33±0.88^{cde}	13.33±2.40^{cd}
CA +AC								
5+5	13.33±0.88^{a}	16.00±3.21^{bcde}	17.67±1.45^{a}	16.33±0.88^{cd}	17.00±0.58^{a}	**18.67±1.86^{b}**	**18.00±1.53^{a}**	14.00±1.53^{cd}
10+10	9.00±1.15^{bc}	11.67±2.33^{def}	15.00±2.08^{ab}	18.00±2.08^{bcd}	12.67±2.33^{ab}	11.33±1.20^{cd}	14.33±2.03^{ab}	13.00±2.08^{cde}
15+15	7.00±0.58^{bcde}	5.33±0.88^{fg}	9.67±1.76^{bcde}	9.33±0.88^{def}	7.00±1.15^{bcd}	4.67±0.88^{de}	8.00±1.15^{bcde}	6.67±1.20^{def}
20+20	3.00±0.58^{ef}	2.00±0.00^{g}	5.67±1.45^{def}	5.50±1.50^{ef}	3.00±0.58^{cde}	1.50±0.50^{e}	4.00±1.15^{ef}	1.00±1.00^{f}

AC-activated charcoal, CA-citric acid, AA-ascorbic acid. Data were collected after two weeks of culture on MS medium fortified with different concentrations of 2,4-D+TDZ along with anti-browning additives. Values are means ± standard error (SE) of three replicated experiments, and those followed by a different letter within a column are significantly different at $p < 0.05$ according to Turkey's test. Best results are showed in bold.

sterilized soil media; the plantlets were covered with transparent polyethylene bags to ensure a high humidity environment for two weeks. Thereafter, plantlets were transferred to unheated glasshouse conditions and grew normally.

Statistical Analysis

The study was carried out according to a completely randomized design with 12 replicates, and each experiment was repeated three times. Controls were maintained at every step of analysis. One-way analysis of variance (ANOVA) in SPSS version 17 software was used for the evaluation of statistical significance, and differences among the means were analyzed by Tukey's test at ($p < 0.05$). The percentage of phenolic secretion was calculated by the following

formula (Kumar et al., 2015).

$$\% \text{ of phenolic secretion} = \frac{\text{number of explants showing phenolic secretion}}{\text{total number of explants}} \times 100$$

Results

Influence of Anti-browning Additives on Phenolic Secretion in Okra Callus Cultures

Phenolic compound secretion from okra explants was observed after 5 d of initial culture. The explants turned brown, which caused the medium to become brownish in color and the explants died after two weeks (Fig. 1a and 1b). The frequency of phenolic compound secretion varied from 0 to 92.2% in hypocotyl and 0 to 88% in cotyledon explants, and the frequency was higher when the anti-browning additives were not added to the medium (Fig. 2a & 2b). Media supplemented with AC showed 0 to 29.7% phenolic secretion from cotyledons, which was less than that in hypocotyl explants (0 to 70.8%). MS medium supplemented with CA alone exhibited increased phenolic secretion. Similarly, phenolic secretion was greater in hypocotyls (20.6 to 77.4%) relative to cotyledon explants (12 to 72.3%) when the medium was supplemented with CA alone (Fig. 2a and 2b). Overall, lower amounts of phenolic secretion were observed when the culture medium was supplemented with CA in combination with AA. Phenolic secretion was also lower (1-20%) in cotyledon than in hypocotyl explants (6-38%). Moreover, callus induction frequency decreased in both types of explants at increased concentrations of anti-browning additives (Fig. 2a and 2b). As a result, callus induction was

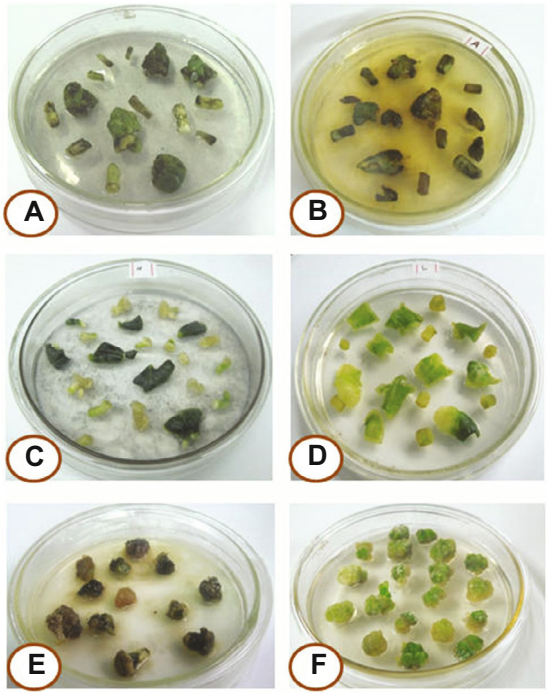

Fig. 1. Influence of anti-browning additives on phenolic secretion and callus response. (A) Initiation of browning of explant after 1st week of culture on anti-browning additives free medium (B) Death of explants after 2nd week on anti-browning additives free medium (C) Explants in culture media supplemented with 200 mg·L^{-1} activated charcoal (D) Explants in culture media supplemented with 5 mg·L^{-1} citric acid + 5 mg·L^{-1} ascorbic acid (E) Death of callus tissue (F) Healthy callus. For (C) and (D) the data were recorded after two weeks of initial culture.

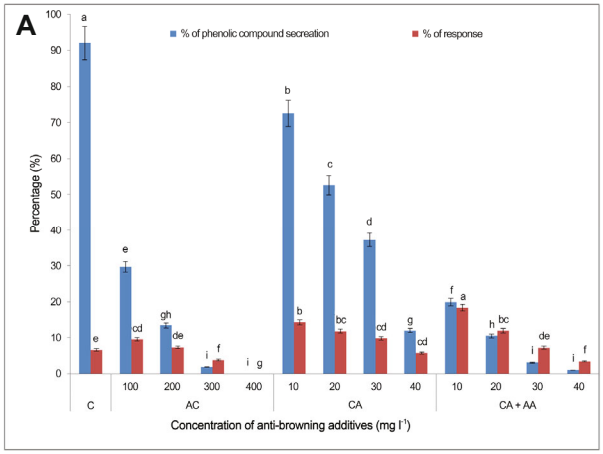

Fig. 2A. Effect of different anti-browning additives on phenolic secretion and callus induction in cotyledon explants.Cotyledon explants cultured on MS medium fortified with different anti-browning additives (10-400 mg·L^{-1}). Data were collected after 2 weeks of culture. Means followed with different letters are significantly different at $p < 0.05$ according to Tukey's test. C - control, AC - activated charcoal, CA - citric acid and AA - ascorbic acid, for CA + AA the combination ratio is 1:1.

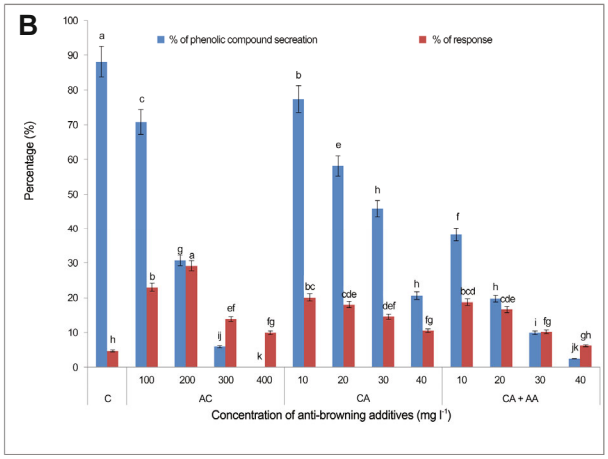

Fig. 2B. Effect of different anti-browning additives on phenolic secretion and callus induction in hypocotyl explants. Cotyledon explants cultured on MS medium fortified with different anti-browning additives (10-400 mg·L^{-1}). Data were collected after 2 weeks of culture. Means followed with different letters are significantly different at $p < 0.05$ according to Tukey's test. C - control, AC - activated charcoal, CA - citric acid and AA - ascorbic acid, for CA + AA the combination ratio is 1:1.

Table 5. Effect of PGRs combinations on shoot differentiation from cotyledon and hypocotyl derived calli in *Abelmoschus esculentus*

Culture media	PGRs mg·L^{-1}				Cotyledon		Hypocotyl	
	BAP	NAA	Zeatin	IBA	Shooting response (%)	Shoot number per calli (Mean ± SE)	Shooting response (%)	Shoot number per calli (Mean ± SE)
SM1	1.5	0.1	-	-	15.33±1.76^{d}	1.33±0.13^{b}	19.67±2.91^{cd}	1.80±0.21^{b}
SM2	2	0.1	-	-	18.67±2.91^{cd}	1.37±0.09^{b}	25.33±2.40^{c}	1.93±0.27^{ab}
SM3	2.1	0.5	-	-	14.67±1.45^{d}	1.24±0.03^{b}	17.33±1.20^{cd}	1.55±0.27^{b}
SM4	1.5	-	-	0.1	27.33±1.76^{bc}	1.68±0.02^{ab}	39.67±3.38^{b}	2.22±0.17^{ab}
SM5	**2**	**-**	**-**	**0.1**	**38.67±2.03^{a}**	**2.05±0.05^{a}**	**52.67±2.91^{a}**	**2.85±0.10^{a}**
SM6	2.5	-	-	0.5	32.00±1.53^{ab}	1.85±0.16^{ab}	45.67±2.33^{ab}	2.05±0.24^{ab}
SM7	-	-	1.5	0.1	12.00±1.73^{d}	1.68±0.22^{ab}	13.33±1.76^{d}	1.97±0.25^{ab}
SM8	-	-	2	0.1	18.33±1.86^{d}	1.35±0.15^{b}	19.00±1.73^{cd}	1.48±0.10^{b}
SM9			2.5	0.5	15.00±0.58^{d}	1.47±0.09^{ab}	15.00±1.73^{cd}	1.39±0.14^{b}

SM represents shooting media, values represents means ± standard error (SE) from three replicated experiments; those followed by same letter within a column are not significantly different at $p < 0.05$ according to Turkey's test.

Table 6. Effect of IBA in combination with activated charcoal (AC) on in vitro rooting of regenerated shoots

Culture media	IBA mg·L^{-1}	AC mg·L^{-1}	Rooting Response (%)	Number of root per explant (Mean ± SE)	Root length (cm) (Mean ± SE)
RM1	1	100	25.67±2.33^{f}	1.33±0.33^{d}	2.07±0.18^{c}
RM2	1.5	100	36.33±2.60^{ef}	1.67±0.33^{cd}	2.63±0.19^{bc}
RM3	2	100	45.67±2.96^{de}	2.67±0.33^{bcd}	3.60±0.31^{ab}
RM4	2.5	100	52.33±3.53^{bcd}	2.33±0.33^{bcd}	3.80±0.23^{ab}
RM5	1	200	50.33±2.40^{cd}	3.10±0.17^{abc}	2.20±0.12^{c}
RM6	1.5	200	63.33±3.71^{abc}	3.67±0.20^{ab}	4.14±0.28^{a}
RM7	**2**	**200**	**76.67±2.40^{a}**	**4.33±0.44^{a}**	**4.67±0.27^{a}**
RM8	2.5	200	65.67±2.33^{ab}	3.40±0.35^{ab}	3.51±0.34^{ab}

RM represents rooting media, values are means ± standard error (SE) from three replicated experiments; those followed by a same letter within a column are not significantly different at $p < 0.05$ according to Turkey's test.

negatively impacted beyond a particular stage. In contrast, the addition of 200 mg·L^{-1} AC and 10 mg·L^{-1} CA+ AA (1:1) to the culture medium was optimal for controlling phenolic secretion in hypocotyl and cotyledon explants and was used for further studies.

Influence of Plant Growth Regulators on Callus Induction

Callus induction was observed after 7-10 days of culture on MS medium fortified with different concentrations of auxins and cytokinins in addition to anti-browning additives (Fig. 1c and 1d). Plant growth regulators including NAA, 2,4-D, BAP, BA, and TDZ were tested for callus induction in cotyledon and hypocotyl explants (Table 1). Hypocotyl explants had a stronger callogenic response that ranged from 1 to 67.7% on different media (Tables 2-4). Moreover, among the 12 tested media, MS + 0.5 mg·L^{-1} 2,4-D + 1.5 mg·L^{-1} BA supplemented with 200 mg·L^{-1} AC provided effective callus induction in hypocotyl explants, with an average of 67.7% of explants producing calli (Table 3). Conversely, cotyledon explants had an overall poor callogenic response (between 0 and 30%); the highest callus response (30%) was observed on MS + 1.5 mg·L^{-1} NAA + 0.5 mg·L^{-1} BAP fortified with 5 mg·L^{-1} CA + 5 mg·L^{-1} AA (Table 2). Overall, the frequency of callus induction was higher in hypocotyl than in cotyledon explants. These results clearly indicate the callus-forming potential of the hypocotyl explants in okra tissue culture.

Interaction Effect of Anti-browning Additives and PGRs on Callus Induction

The frequency of callus formation varied between okra explant types and medium compositions (Tables 2-4). For media supplemented with AC, callus formation efficiency was lower in cotyledon (0-14.6%) than hypocotyl explants (1-67.7%) for all combinations of PGRs (Tables 2-4).

Moreover, addition of AC induced rooting in hypocotyl explants on MS media containing NAA and BAP, which in turn prevented callus formation in the surviving explants. In contrast, the combination of 2,4-D and BA (1:3) in the medium favored callus formation in hypocotyl explants when AC was also added. The same medium formulation increased the frequency of callus formation to the highest level (i.e. 67.7%) for hypocotyls (Table 3). Our results showed that CA alone was not effective at reducing phenolic secretion; however, medium supplemented with CA resulted in greater callus formation in cotyledon than hypocotyl explants on all tested PGRs combinations (Tables 2-4). Cotyledon explants yielded the highest callus induction (30%) on medium supplemented with lower concentrations of CA + AA and NAA and BAP, whereas the same medium formulation resulted in less callus induction in hypocotyl explants (Table 2). These results clearly indicate that anti-browning additives in the culture medium significantly altered the ratio of medium components and consequently influenced callus induction of both explants.

Callus Growth and Development

The transfer of calli to media supplemented with anti-browning additives negatively affected their growth, morphology, and color (data not shown). In such cases, the callus tissue became soft, watery, and non-reproductive. To avoid these negative effects of anti-browning additives, formed calli were cultured on MS medium supplemented with different auxins and cytokinins without the addition of anti-browning additives. Among the auxin and cytokinin combinations, 1 $mg \cdot L^{-1}$ NAA + 0.5 $mg \cdot L^{-1}$ BAP induced the best callus growth and development (data not shown). Rapid growth and soft, yellowish-green texture were observed for calli from both explant sources (Fig. 1h). Calli were subcultured on fresh medium every two weeks. In general, the calli became granular and ready for regeneration at 1.5 months after explant inoculation of the culture medium.

Shoot Regeneration

Soft, yellowish-green calli were transferred to shoot regeneration media containing full-strength MS medium supplemented with various combinations and concentrations of auxins and cytokinins (Table 5). After four weeks of culture, leaf primordia were observed in the calli (Fig. 3c and 3d). Between the explant types, 52.6% of hypocotyl-derived calli showed efficient in vitro shoot regeneration and produced 2.8 shoots/calli, which was the highest number produced in our cultured conditions. The cotyledon-derived calli resulted in 38.6% of shoot regeneration with 2.0 shoots/calli (Table 5). Moreover, among the nine tested media conditions, MS + 2 $mg \cdot L^{-1}$ BAP + 0.1 $mg \cdot L^{-1}$ IBA (SM5) produced the best level of shoot formation and average number of shoots per

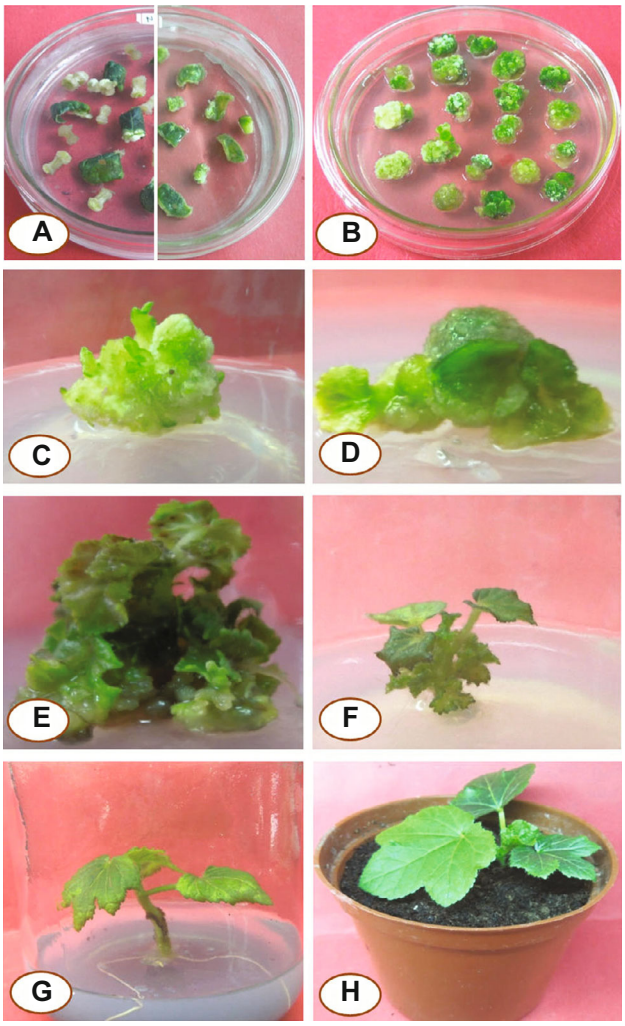

Fig. 3. Callogenesis and organogenesis of *Abelmoschus esculentus*. L. (A) Callus formation from hypocotyl (left side) and cotyledon (right side) explants. (B) Healthy callus after proliferation. (C) Shoot organogenesis from hypocotyl calli. (D) Shoot organogenesis from cotyledon calli. (E) and (F) Shoot elongation. (G) Rooting. (H) Acclimatized okra plant in culture room.

callus in both types of explant-derived calli.

Rooting and Acclimatization of Plantlets

The best in vitro root induction was observed on half-strength MS media supplemented with AC and IBA. Rooting medium without AC resulted in slower root induction and fewer roots (data not shown), whereas the addition of AC accelerated the root induction and improved the quality and quantity of roots. Root induction was greater (76.6%) in ½ MS medium + 2 $mg \cdot L^{-1}$ IBA and 200 $mg \cdot L^{-1}$ AC (RM7). On the same medium, higher average number of roots (4.33) and the longest roots (4.67 cm) were acquired within 1 month of culturing (Table 6; Fig. 3g). After removal of the plantlets from MS medium, the roots were washed with sterile water and the plantlets were transferred to sterile pots containing vermiculite and sand (3:1) with sufficient moisture. Plantlets

were covered with transparent polyethylene bags and nurtured in an environmentally controlled room under 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light and 25 ± 2°C for three weeks. During the acclimatization period, the leaves turned black-green and expanded in size (Fig. 3h). The survival rate of the acclimatized plantlets after transfer to the greenhouse was 80%.

Discussion

In vitro regeneration of okra is attained mainly through callus formation. To date, however, few studies have reported regeneration through calli in this species. Factors such as medium texture (Anisuzzaman et al., 2010), explant type (Kabir et al., 2008), and phenolic compound secretion into the medium (Narendran et al., 2013) likely affect okra regeneration. Our findings revealed that anti-browning additives and manipulation of plant growth regulators in the culture medium affect phenolic compound secretion and callus induction frequency from both okra hypocotyl and cotyledon explants.

Phenolic compounds are mostly secreted into the medium from cut parts of explants, causing browning of medium and explant death. These phenomena are a major problem in okra tissue culture and hinders its regeneration (Manickavasagam et al., 2015). This phenomenon, which is caused by the oxidation of phenolic compounds, negatively affects the nutrient uptake efficiency of explants, leading to plant death (Jones and Saxena, 2013). To inhibit phenolic secretion and reduce explant browning during plant tissue culture, compounds such as AC, CA, and AA are added to the culture medium (Çördük and Aki, 2011; Erland and Mahmoud, 2014).

AC adsorbs phenolic compounds and renders PPO and POD inactive, preventing tissue browning (Pan and Staden, 1998; Thomas, 2008). Indeed, AC has been used to inhibit browning in many plant species, including *Phoenix dactylifera* (Meziani et al., 2016), *Lavandula* x *intermedia* (Erland and Mahmoud, 2014) and *Sorghum bicolor* (Nguyen et al., 2007). Similarly, CA and AA have been used to prevent exudation of browning compounds into the medium from various plant species, including *Zingiber officinale* (Sundram et al., 2012) and *Vicia faba* (Klenotičová et al., 2013). The supplement, citric acid contains citrate, which works as a chelating agent that prevents phenolic secretion and subsequent browning of tissues (Pankaj et al., 2014). On the other hand, AA scavenges oxygen radicals produced during explant injury, thereby protecting plant cells from oxidative harm (Titov et al., 2006). In a study by Ko et al. (2009), AA not only minimized phenolic secretion but also reduced tissue browning in affected plantlets of in vitro-grown Cavendish banana. Our results show that supplementing the culture medium with anti-browning additives was effective at controlling lethal browning in okra tissue culture; however, the efficiency of this medium supplement was dependent on the type of explant and PGRs used. For example, different combinations of anti-browning additives and PGRs affected callus induction in both explant types differentially (Tables 2-4). In our study, addition of AC to the medium inhibited callus formation from cotyledon explants with all tested PGRs, whereas rhizogenesis was induced in hypocotyl explants specifically with the NAA and BAP combination. Our results are consistent with the findings of Klenotičová et al. (2013), who showed that AC induces rhizogenesis in explants during shoot apex culture of *V. faba* but inhibits callus induction in *Gossypium hirsutum* (Zhang et al., 2001) and S*orghum bicolor* (Nguyen et al., 2007). These negative effects may be due to the non-selective adsorption of essential components of the culture medium by AC (Thomas, 2008). Dumas and Monteuuis (1995) reported that AC adsorbs BAP from media, which makes the media darker and simulates soil conditions that promote the rooting of explants. Thomas (2008) showed that AC in culture media can significantly alter the ratio of media components and consequently influence plant regeneration. In the present study, it was clear that 2,4-D and BA (1:3) were necessary for callus formation from hypocotyl explants when 200 $mg \cdot L^{-1}$ AC was included in the culturing conditions. This suggests that the high cytokinin and low auxin proportions with AC in the medium were beneficial for callus induction from hypocotyl explants. We observed the highest percentage of callus induction (30%) from cotyledon explants on medium supplemented with lower concentrations of CA + AA in combination with NAA + BAP (3:1). It is known that AC induces cell division, cell differentiation, and cell elongation (Ko et al., 2009; Smirnoff, 1996). Klenotičová et al. (2013) also reported reduced phenolic secretion and high callus formation from cotyledonary nodes of *V. faba* on MS medium supplemented with CA + AA. In another study in *Litchi chinensis*, Pankaj et al. (2014) used AA as a vitamin source to initiate the proliferation of cells of explants as well as a browning control agent. Similarly, in the present study CA + AA were effective antioxidants in callus formation to avoid phenolic secretion from cotyledon explants of the Wufu okra cultivar. Thus, our findings show that combining CA and AC could be a useful method to control tissue browning and cell proliferation in explants.

In previous studies, a high frequency of callus induction from hypocotyls in okra was attained on medium supplemented with NAA + TDZ (Anisuzzaman et al., 2010) and with NAA + BAP (Kabir et al., 2008). In our study, hypocotyl explants had more callus initiation relative to that from cotyledon explants. However, our findings contradict previous reports regarding the PGRs combinations for callus initiation from hypocotyl and cotyledon explants. Differences in explant responses to various PGRs can be explained based on their differential reactivity to media components (Ikram-

ul-Haq, 2005), endogenous hormone levels (Kumar and Srivastava, 2015), and genotype (Gerszberg et al., 2015). We hypothesize that explant type and medium components played important roles in okra callus initiation in our study.

We examined cytokinins in combination with auxins for shoot induction from hypocotyl and cotyledon explants (Table 5). Previous studies showed that BAP in combination with low concentrations of auxins resulted in high shoot regeneration frequency in *Solanum melongena* (Satish et al., 2015), *Brassica juncea* (Guo et al., 2005), and *Brassica napus* (Maheshwari et al., 2011). In our study, 2 mg·L^{-1} BAP + 0.1 mg·L^{-1} IBA produced the highest percentage of shoots from both types of explant-derived calli (Table 5). Similarly, Anisuzzaman et al. (2010) reported the highest shoot regeneration (65%) from hypocotyl-derived calli in the okra cultivar BARI Dherosh-1 using the same combinations of BAP and IBA. Moreover, efficient shoot regeneration from hypocotyl-derived calli is well documented in *Brassica oleracea* (Gerszberg et al., 2015), *S. melongena* (Muthusamy et al., 2014), and *Cucurbita pepo* (Pal et al., 2007). Explant- dependent morphogenesis in plant tissue culture is due to differences in the uptake of nutrient elements (Razdan, 2003) or the regeneration capability of individual explants (Sharma and Rajam, 1995). The findings from the present study also support the previous reports of the variance in the totipotency of different explants from the same plant source. Therefore, the selection of explants is a necessary step for an efficient regeneration system.

Poor in vitro rooting during tissue regeneration is a major obstacle for successful micropropagation in many plant species (Dewir et al., 2016; Oakes et al., 2016). In previous studies, reduced salt concentration (1/2 MS) in the medium significantly increased root induction in *Allium cepa* (Ramakrishnan et al., 2013) and *Solanum. melongena* (Satish et al., 2015). The addition of AC was also shown to significantly enhance root formation in *Simmondsia chinensis* (Bala et al., 2015) and *Jatropha curcas* (Rather et al., 2011). Therefore, for successful rooting of in vitro-derived shoots, we used ½ MS medium supplemented with AC and IBA. The reduced salt concentration is thought to be effective for in vitro rooting due to the reduced nitrogen content rather than reduced osmotic potential; however, AC stimulates nitrogen uptake by the shoots and provides a dark environment, which induces in vitro rooting (Thomas, 2008). In our study, the greatest amount of rooting was obtained on ½ MS medium with reduced salt that was fortified with 200 mg·L^{-1} AC + 2 mg·L^{-1} IBA. A similar correlation between AC and IBA was reported by Swamy et al. (2014) in *Pogostemon cablin* Benth and by Rathore et al. (2015) in *J. curcas*. Our results show that adding AC and IBA in half-strength MS medium can improve the in vitro rooting protocol for okra.

We have established an efficient in vitro plant regeneration protocol for the *A. esculentus* cultivar Wufu by reducing excessive phenolic secretion from hypocotyl and cotyledon explants in the culture medium through the addition of additives and manipulation of plant growth regulators. Hypocotyl explants were the best for callus initiation and shoot regeneration. The addition of AC was an efficient anti-browning agent for callus initiation from hypocotyl explants in media containing 2,4-D and BA (1:3), whereas CA + AA at low concentrations with the addition of NAA and BAP (3:1) was beneficial for high callus induction from cotyledon explants. The combination of BAP and IBA in the culture medium was effective for shoot induction from both explants. Thus, the rooting protocol for okra explants was improved by the supplementation of AC and IBA in half-strength MS medium. This regeneration protocol may be beneficial for future breeding and transformation programs of *A. esculentus.*

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