Micropropagation of *Agave americana*

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Abstract. *Agave* species are economically important plants in tropical and subtropical desert ecosystems as ornamentals as well as potential bioenergy crops. However, their relatively long life cycles and the current lack of biotechnology tools hinder their breeding. In this study, an efficient system for micropropagation was developed for *Agave americana* L. by using basal stems as explants and grown on a modified Murashige and Skoog medium (MSI) or a 1/2 MSI medium supplemented with various concentrations of 6-benzylaminopurine (BA) for shoot proliferation. The highest number of shoots (18.5 shoots/explant) from basal stems was obtained on MSI supplemented with 13.32 μM BA. An efficient shoot regeneration system was also developed from leaf tissues. Combinations of auxin with cytokinin, basal media, and leaf regions were optimized for shoot induction. Adventitious shoot formation from leaf segments was induced and proliferated with combination ranging of 0.54 to 2.68 μM [α -naphthaleneacetic acid (NAA)] with 8.88 to 13.32 μM (BA), and the maximum frequency ($\approx 69\%$) was obtained with 2.68 μM NAA plus 13.32 μM BA. MSI medium and the basal segment of leaf affected shoot induction. The highest rooting frequency and mean number of shoots occurred in 1/2 MSI containing with 4.92 μM indole-3-butyric acid (IBA) alone (90%, 3.4) or 1.48 μM IBA plus 1.61 μM NAA (92%, 5.2). Survival of in vitro plantlets after transfer and acclimatization to ex vitro conditions was 87%. This is the first complete protocol for micropropagation of *A. americana*.

Agave species (*Agave* L.) are important species in tropical and subtropical ecosystems and are also cultivated as ornamentals. They have been recently recognized as highly promising bioenergy plants because they yield high cellulosic biomass while using water three to six times more efficiently than any of the current candidate crops for biomass

(Somerville et al., 2010). They are also adapted to marginal lands with low fertility (Somerville et al., 2010). As a result of these features, agaves are potentially suitable for production as bioenergy feedstocks in $\approx 18\%$ of the semiarid terrestrial surface, which typically has from 200 to 800 mm of rainfall per year and an average growing season temperature of less than 21 $^{\circ}\text{C}$ (Somerville et al., 2010).

However, despite their significance and potential, relatively little genomics, functional genomics, and biotechnological research have been done with agaves for bioenergy feedstock use. Its long life cycle has become an obstacle to conventional breeding (Portillo et al., 2007). Therefore, the most effective means for genetic improvement is through biotechnology. Micropropagation systems and regeneration systems through either organogenesis or somatic embryogenesis have already been established for a few *Agave* species

(Martínez-Palacios et al., 2003; Portillo et al., 2007; Santacruz-Ruvalcaba and Portillo, 2009). Shoots have been induced using bulb, rhizome, stem, and leaf explants in *A. arizonica* Perrine ex Engelm (Powers and Backhaus, 1989), *A. fourcroydes* Lemaire (Robert et al., 1987), and *A. sisalana* Perrine ex Engelm (Das, 1992; Hazra et al., 2002); using leaf segments and meristematic tissue in *A. tequilana* F.A.C Weber (Valenzuela-Sánchez et al., 2006); and using axillary shoots in *A. parrasana* A. Berger (Santacruz-Ruvalcaba et al., 1999). Somatic embryogenesis (SE) has also been reported in *A. fourcroydes* using apical meristems (González et al., 2003), in *A. victoriae-reginae* T. Moore using stem segments (Martínez-Palacios et al., 2003), in *A. salmiana* Otto ex Salm-Dyck using stems (Flores-Benítez et al., 2007), in *A. tequilana* using leaves (Portillo et al., 2007, 2012; Rodríguez-Sahagún et al., 2011; Santacruz-Ruvalcaba and Portillo, 2009), and in *A. sisalana* using young leaves (Nikam et al., 2003). However, shoot propagation, SE, and plant regeneration of *A. americana* have not been reported.

The aim of this work was to establish a high-frequency shoot micropropagation and plant regeneration protocol for *A. americana*, a giant agave native to Central America, which can grow in arid, semiarid, tropical, and subtropical regions such as the southern United States, North Africa, Mexico, India, and southern China (Jin et al., 2003, 2004; Lachenmeier et al., 2006; Nasri and Ben Salem, 2012). Recently, some researchers have evaluated *Agave* as a bioenergy feedstock because of its stem carbohydrates and leaf lignocelluloses (Debnath et al., 2010; García-Moyae et al., 2011; Jaouadi et al., 2011; Somerville et al., 2010). The efficient protocols of micropropagation and regeneration developed in this research will benefit future clonal propagation, breeding, and biotechnology-based genetic improvement.

Materials and Methods

Plant materials and in vitro germination.

Seeds of *A. americana* (purchased from CactusStore.com; <<http://www.cactusstore.com>>) were soaked in distilled water for 24 h. They were surface-sterilized in 70% (v/v) ethanol for 2 min, then immersed in 20% (v/v) commercial bleach (1.5% NaOCl) with several drops of Tween 20 for 20 min, and followed by rinsing three times with sterile distilled water. After blotting them on sterile filter paper to remove excess water, they were planted in 150 $\times$ 20-mm culture tubes containing 20 mL Murashige and Skoog (1962) (MS) basal medium supplemented with 30 g·L⁻¹ sucrose, 7 g·L⁻¹ agar, pH 5.8 for germination. Leaves and basal stems of 6-week-old seedlings were used as explants and incubated with a 16-h photoperiod at 25 $\pm$ 1 $^{\circ}\text{C}$ with 55 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ fluorescent illumination.

Culture medium and culture conditions.

Four media used in this study were: MS medium, modified MS medium (MSI)

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containing MS basal salts (Sigma-Aldrich; M5524), 250 mg·L⁻¹ 2-(N-morpholino) ethane sulfonic acid, 200 mg·L⁻¹ L-glutamine, 100 mg·L⁻¹ myo-inositol, and 100 mg·L⁻¹ each of nicotinic acid, pyridoxine HCl, calcium pantothenate, thiamine HCl, and L-cysteine, Driver and Kuniyuki Walnut (DKW) medium (Driver and Kuniyuki, 1984), and Woody plant medium (WPM; Lloyd and McCown, 1981). All media were supplemented with 30 g·L⁻¹ sucrose and adjusted to pH 5.8 with 1 M KOH before the addition of 7 g·L⁻¹ agar and then autoclaved for 20 min at 121 °C. All plant growth regulators (PGRs) (see specific experiments) were filter-sterilized into media after autoclaving unless stated otherwise. Cultures were incubated at 25 ± 1 °C with a 16-h photoperiod using 55 μmol·m⁻²·s⁻¹ fluorescent illumination.

Shoot multiplication from explants of basal stems. Basal stems, ≈0.3 cm long, were isolated from 6-week-old defoliated seedlings and transferred onto MSI or 1/2 MSI (1/2 MS basal salts) media supplemented with 2.2, 4.4, 8.8, or 13.2 μM of BA for inducing axillary shoots. Explants were placed in 200 mL (9.5 cm high × 4.5 cm diameter) baby food jars containing 50 mL of medium. Microcuttings were transferred to fresh media every 30 d. The longest leaves were measured after 30 d and the numbers of shoots were recorded after 30 and 50 d, respectively. The gain of fresh weight (FW) after 30 d was determined as follows:

$$\text{FW gain(g)} = \text{explant weight after 30 d of culture(g)} - \text{initial weight(g)}$$

Shoot regeneration from leaf explants. To determine the effect of PGRs on shoot induction, leaves from 6-week-old plantlets were cut into ≈0.5-cm long sections and incubated on MSI medium containing 0, 2.22, 4.44, 8.88, 13.32 μM BA in all combinations with 0, 0.54, 2.68, 5.36 μM NAA. Leaf segments were placed in petri dishes (1.5 × 8.5 cm) containing 20 mL medium. Explants were subcultured to the same fresh medium every 30 d. Explant morphology, callus induction frequency, and percentage of explants forming shoots and shoot numbers were recorded after 30, 60, 90, and 120 d.

To test how different leaf regions affected shoot formation, leaves (≈3 cm long) were cut transversely into five segments: one basal, two middle, and two apical. These segments were placed onto MSI in sequential order and media were supplemented with 2.68 μM NAA and 13.32 μM BA or 5.34 μM NAA and 4.44 μM BA. Each treatment had three replicates per replication with 16 leaf segments.

Histology light microscopy. For histology, samples were fixed in formalin, acetic acid, alcohol solution (FAA) containing 70% ethanol, 5% glacial acetic acid, and 5% formaldehyde at room temperature for 24 h. After fixation, samples were dehydrated with a graded series of isopropanol (50%, 75%, 85%, 95%, and 100%) and then embedded in Paraplast Plus blocks (58 to 60 °C). Sections (10-μm thick) were obtained using a micro-

tome and stained with safranin O, crystal violet, and fast green (Jensen, 1962). The sections were viewed using a microscope (Olympus BX51; <<http://www.olympusamerica.com>>) equipped with a digital camera (Olympus Q-Color 5; <<http://www.olympusamerica.com>>) for photomicrography.

Rooting induction from microshoots. The effects of IBA and NAA and their concentrations on root induction from plantlets were examined. Shoots separated from clusters on basal stems or on leaf segments were cultured in 1/2 MSI medium supplemented with 0, 0.49, 1.48, 2.46, or 4.92 μM IBA alone; 0, 0.54, 1.61, 2.68, or 5.36 μM NAA alone; or a combination of IBA with NAA (1.48 + 2.68, 2.46 + 2.68, or 4.92 + 5.36 μM, IBA + NAA) (Table 5). Rooting percentage, root number, and maximum root length were recorded after 10 and 20 d in culture.

Acclimatization. Plantlets ≈3 to 4 cm high with healthy roots were gently washed in tap water to remove agar residue and transplanted to wide pots (10 × 8.5 cm) containing PRO-Mix soil (Premier Horticulture, Quebec, Canada). Pots were covered for 2 d with transparent polystyrene jars with holes to maintain high humidity. Plants were watered with inorganic salt solution [nitrogen:15%, phosphorus (P₂O₅):16%, potassium (K₂O):17%, magnesium: 0.055%, boron: 0.02%, copper: 0.0075%, iron: 0.075%, manganese: 0.042%, molybdenum: 0.0075%, zinc: 0.012%; Peters® System of Plant Nutrition, Scotts-Sierra Horticultural Products Company, Marysville, OH) once daily for the first 2 d and every other day thereafter and grown in a growth chamber (85% humidity, 25 ± 1 °C, 16-h photoperiod with 55 μmol·m⁻²·s⁻¹ fluorescent light). Plant survival was recorded after 6 weeks.

Data analysis. Experiments were repeated three times. Each experiment was performed using eight replicates (bottles) with three explants each for shoot proliferation from basal stems, three replicates (dishes) with 16 leaf segments each for shooting from leaves, and 10 replicates (bottles) with two explants each for rooting. Data were subjected to analysis of variance or regression analysis, and the means ± SES was calculated using Duncan's multiple-range test (Duncan, 1955) with SPSS 17.0 Version (<<https://www14.software.ibm.com>>). *P* values ≤ 0.05 or 0.01 were considered to represent significant differences among treatments.

Results

Shoot proliferation from basal stem regions. Media (MSI or 1/2 MSI), BA, and media × BA interaction (expect culture 30 d) all had a significant effect on number of shoots per explant (*P* < 0.05 or *P* < 0.01) (Table 1). There was a significant positive linear correlation between BA concentration and shoots number per explant (*P* < 0.01), whereas media composition had no significant linear correlation (*P* > 0.05) by regression analysis. BA concentration affected shoot formation more than the composition of basal medium did (*r*_{BA} > *r*_{Media}).

Media (MSI or 1/2 MSI) supplemented with BA (2.22 to 13.32 μM) alone or in combination with 0.54 μM NAA (data not presented) produced healthy shoots without calli in the basal stem area (Fig. 1A–B). However, calli occurred at shoot bases on medium containing NAA greater than 2.68 μM (Fig. 1C) (data not presented). Optimum shoot formation after 50 d (18.5 shoots/explant in MSI; 13.4 shoots/explant in 1/2 MSI) occurred on media containing 13.32 μM BA and was significantly higher than on media with other BA concentrations (*P* < 0.001) (Table 1).

Regression analysis showed media composition and BA concentration had a significant positive linear correlation on FW gain (*P* < 0.01), whereas no linear correlation was found on the longest leaf length (*P* > 0.05) (Table 1). Media × BA interaction on leaf length and FW gain, BA concentration on leaf length, and media composition on FW gain all has a significant effect in shoots of *A. americana* (*P* < 0.01 or *P* < 0.05) (Table 1). Half-strength MSI supplemented with 8.8 or 13.32 μM BA yielded the greatest FW gain (1.7 g) in each shoot (Table 1). Additionally, leaf length was significantly greater in shoots produced on MSI medium with 2.22 μM BA or on 1/2 MSI with 13.32 μM BA than on media with other PGR treatments (*P* < 0.001) (Table 1).

Callus, shoot induction, and proliferation from leaf segments. Callus, shoot induction, and proliferation were achieved from leaf segments by culturing them on MSI media supplemented with combinations of BA and NAA (Fig. 2A). No shoots were obtained on MSI basal medium without PGRs (control). Different combinations of NAA and BA induced shoot formation (Table 2). Explants placed on media with BA (2.22 to 13.32 μM) alone or BA (0.54 μM) and NAA (2.22 to 4.44 μM) did not grow or survived initially and then became necrotic. No callus or shoots formed on these explants (Table 2; Fig. 2B).

Calli initiated from the cut edges of 14.6% to 64.6% of explants by 30 d of culture on media containing NAA (0.54 to 2.68 μM) and BA (8.88 to 13.32 μM). One or two adventitious shoots regenerated directly from leaf segments cultured on media with 0.54 μM NAA and 8.88 μM BA and 0.54 μM NAA and 13.32 μM BA after 45 d, and these adventitious shoots proliferated an average of 12.5 shoots on medium containing 0.54 μM NAA and 8.88 μM BA after 90 d (Fig. 2C–D). Green calli formed from leaf segments on medium supplemented with 2.68 μM NAA and 8.88 or 13.32 μM BA and became white after 60 d. Meanwhile, the explanted leaf segment swelled and by 60 d of culture became compact, smooth, and two to five times the initial size (Fig. 2E). After this time, the leaf segment turned to a lustrous green and developed many deep green spherical masses by 90 d (Fig. 2F). After an additional 1 to 2 weeks, these masses developed into shoot clusters (Fig. 2G–H) and produced a mean of 23.4 adventitious shoots (AdS) per explant. Secondary shoots from adventitious shoots were observed after 140 d of

Table 1. Effects of 6-benzylaminopurine (BA) concentration on *Agave americana* proliferation from basal stems.^z

Media	BA (μM)	Shoots per explant		Longest leaf length (cm)	FW ^y gain (g/explant)
		After 30 d	After 50 d	After 30 d	After 30 d
MSI	2.22	2.0 ± 0.4 c	6.0 ± 0.4 d	6.6 ± 0.7 a	0.8 ± 0.1 c
	4.44	6.0 ± 0.4 b	10.8 ± 1.5 bc	4.4 ± 0.3 b	1.2 ± 0.2 abc
	8.88	6.8 ± 0.6 b	14.0 ± 3.1 ab	5.2 ± 0.3 ab	1.0 ± 0.1 abc
	13.32	9.3 ± 0.7 a	18.5 ± 1.9 a	5.0 ± 0.3 b	1.1 ± 0.4 abc
1/2 MSI	4.44	5.3 ± 0.5 b	6.8 ± 0.6 d	4.0 ± 0.3 b	0.9 ± 0.3 bc
	8.88	5.2 ± 0.5 b	10.0 ± 0.9 bc	4.9 ± 0.2 b	1.7 ± 0.5 a
	13.32	6.3 ± 0.3 b	13.4 ± 0.5 ab	6.6 ± 0.5 a	1.5 ± 0.7 ab
Analysis of variance ^x					
P value	Media	0.0003**	0.0476*	0.2848 NS	0.0007**
	BA	0.0000**	0.0000**	0.0000**	0.0943 NS
	Media × BA	0.0744 NS	0.0087*	0.0261*	0.0002**
Regression analysis ^x					
BA-linear	p	0.0000**	0.0000 **	0.1147 NS	0.0159*
	r ^w	0.665	0.697	0.2186	0.3804
Media-linear	p	0.2905 NS	0.0753 NS	0.3506 NS	0.0064**
	r ^w	-0.1013	-0.260	-0.0705	0.4354

^zMeans within a single column followed by different letters within the same column represent significant difference ($P \leq 0.001$) by the Duncan's multiple range test. Correlation of media, BA, and m × BA interaction to shoots/explant, longest leaf length, and FW gain was performed by bivariate analysis and linear regression in SPSS 17.0 (SPSS Inc., Chicago, IL).

^yFW = fresh weight.

^xNS, *, ** Nonsignificant or significant at $P \leq 0.05$ or ≤ 0.01 , respectively.

^wr = correlation coefficient of regression analysis.

MSI = modified Murashige and Skoog medium.

Fig. 1. Shoot formation and development from basal stem of *A. americana* L. (A–B) Shoot formation from basal stems in medium with BA alone. (C) Callus formed from shoot base in medium with BA and NAA. BA = 6-benzylaminopurine; IBA = indole-3-butyric acid; NAA = α -naphthaleneacetic acid.

culture and roots on shoot clusters were occasionally found (Fig. 2I–K).

A higher frequency of explants (95.6%) formed calli on media with NAA (2.68 to 5.36 μ M) and BA (2.22 to 13.32 μ M), especially on media with 5.36 μ M NAA and 4.44 to 13.32 μ M BA, after 30 d, and were significantly higher than those on explants cultured on other media ($P < 0.01$) (Table 2). Calli formed on media with 5.36 μ M NAA and 8.88 to 13.32 μ M BA grew vigorously and were crumbly and loose after 60 d. Hairy roots grew from green callus regions, and afterward, these calli frequently browned and became necrotic. In contrast, green calli formed on media containing 2.68 or 5.36 μ M NAA combined with 4.44 μ M BA that became yellowish tissue by 60 d and subsequently produced many small deep green cell masses dispersed in loose, yellowish calli after

90 d. These green masses differentiated into visible adventitious shoots by 120 d (Table 2). The highest frequency and number of adventitious shoots per explant were observed on media with 2.68 μ M NAA and 4.44 μ M BA (52.3%) ($P < 0.01$).

Analysis of variance indicated BA, NAA, and BA × NAA interaction had significant effects on explant survival, callus response, AdS, and number of AdS per explant ($P < 0.01$). Regression analysis showed that there was a positive linear correlation between NAA concentration and callus response, AdS formation and number of AdS per explant ($P < 0.01$ or $P < 0.05$), or between BA concentration and explant survival ($P < 0.01$ or $P < 0.05$) (Table 2).

Explant regions along the leaf for shoots regeneration. Different leaf segments (basal, middle, or apical) from 6-week-old seedlings

had significantly different capacities ($P < 0.001$) for forming calli and adventitious shoots. Basal segments generated the highest percentage and mean number of shoots per explant (Table 3). These capacities decreased along with the distance of the explant from the leaf base (Table 3).

Basal medium for shoot regeneration. Four basal media, all augmented with 2.68 μ M NAA and 13.32 μ M BA, significantly affected the frequency of adventitious shoot formation from leaf segments and number of adventitious shoots per explant (Table 4). No adventitious shoots formed on WPM medium, whereas 68.8% of explants grown on MSI medium produced adventitious shoots with 23.4 shoots per explant. This yield was significantly more than those cultured on MS medium (43.8% explants produced an average of 15.8 shoots per explant). Explants grown on

Fig. 2. Adventitious shoots regeneration from leaf explants of *A. americana*. (A) Leaf explants, (B) leaf explant became brown and necrotic in medium without NAA, (C–D) direct shoot regeneration from leaf explants with NAA + BA at 0.54 + 8.88 and 0.54 + 13.32 (μM + μM) after 45 d. (E–H) Adventitious shoots developed in medium with NAA + BA at 2.68 + 13.32 (μM + μM), leaf segments welled after 80-d culture (E), visible shoots after 90-d culture (F), shoot clusters from one leaf segment (G–H) for 120-d culture. (I–K) one cluster shoot (I), secondary shoots (J), root formation on some shoots (K) for 140-d culture. NAA = α -naphthaleneacetic acid; BA = 6-benzylaminopurine.

MS medium shoots yielded significantly higher number of shoots than those cultured on DKW medium (14.3% explants produced average of eight shoots per explant) ($P < 0.01$).

Histological observation. Histological examination of cultured explants confirmed that direct shoot formation was the method of

regeneration. Shoots were attached directly to explants tissue by a well-developed vascular cylinder and without an intervening callus (Fig. 3A). Typically the structure of monocot shoot apical meristems and associated primordial leavers was observed in all sections of regenerated shoots (Fig. 3B).

Rooting and plant formation. Shoots (3 to 4 cm in length) were excised from shoot clusters and transferred to rooting medium. On 1/2 MSI root medium with different concentrations of NAA and IBA, shoots initiated adventitious roots after 10 d of culture. Rooting percentages and mean numbers of

Table 2. Effects of α -naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) on callus and adventitious shoot formation in *A. americana* leaf segments on MSI medium.^z

NAA (μ M)	BA (μ M)	Callus response (%) after 30 d	Explant survived (%) after 60 d	AdS ^y (%) after 90 d	AdS ^y number per explant after 120 d
0	0	0.0 g	14.6 $\pm$ 2.1 a	Necrosis	0.0 f
0	2.22	0.0 g	18.8 $\pm$ 3.6 a	Necrosis	0.0 f
	4.44	0.0 g	58.3 $\pm$ 5.5 b	Necrosis	0.0 f
	8.88	0.0 g	66.7 $\pm$ 4.2 bc	Necrosis	0.0 f
	13.32	0.0 g	89.6 $\pm$ 2.1 d	Necrosis	0.0 f
0.54	2.22	10.4 $\pm$ 2.1 f	75.0 $\pm$ 7.2 bc	Necrosis	0.0 f
	4.44	12.5 $\pm$ 3.6 f	81.3 $\pm$ 3.6 bc	Necrosis	0.0 f
	8.88	14.6 $\pm$ 2.1 f	75.0 $\pm$ 3.6 bc	12.5 $\pm$ 3.6 d	7.0 $\pm$ 0.6 e
	13.32	27.1 $\pm$ 4.2 e	81.3 $\pm$ 3.6 bc	6.3 $\pm$ 0.0 e	11.3 $\pm$ 1.5 de
2.68	2.22	77.1 $\pm$ 2.1 c	83.3 $\pm$ 7.5 bc	8.3 $\pm$ 2.1 e	14.3 $\pm$ 1.4 cd
	4.44	79.2 $\pm$ 2.1 bc	85.4 $\pm$ 5.5 bc	52.3 $\pm$ 2.1 b	25.0 $\pm$ 0.8 a
	8.88	39.6 $\pm$ 5.5e	87.5 $\pm$ 3.6 d	23.0 $\pm$ 3.6 c	15.3 $\pm$ 0.9 cd
	13.32	64.6 $\pm$ 2.1 d	91.7 $\pm$ 2.1 d	68.8 $\pm$ 3.6 a	23.4 $\pm$ 2.7 ab
5.36	4.44	95.8 $\pm$ 2.1 a	89.6 $\pm$ 2.1 d	35.4 $\pm$ 3.5 c	18.5 $\pm$ 2.2 b
	8.88	87.5 $\pm$ 3.6 ab	75.0 $\pm$ 3.6 d	6.35 $\pm$ 3.6 e	9.8 $\pm$ 0.9 e
	13.32	91.7 $\pm$ 2.1 a	60.4 $\pm$ 5.5 b	Necrotic	0.0 f
Analysis of variance ^x					
P value	NAA	0.0000**	0.0000**	0.0000**	0.0000**
	BA	0.0000**	0.0000**	0.0003**	0.0000**
	NAA $\times$ BA	0.0000**	0.0000**	0.0000**	0.0000**
Regression analysis ^x					
NAA-linear	P value	0.0000**	0.1085 NS	0.0000**	0.0000**
	r ^w	0.914	0.176	0.571	0.502
BA-linear	P value	0.1351 NS	0.0152*	0.5190 NS	0.1399 NS
	r ^w	0.172	0.304	-0.024	0.135

^zMeans within a single column followed by different letters within the same column represent significant difference ($P < 0.01$) by Duncan's multiple range test. Correlation of NAA, BA, and media NAA $\times$ BA to callus response, explant survival, AdS formation, and no. of AdS per explant were performed by bivariate analysis and linear regression in SPSS 17.0 (SPSS Inc., Chicago, IL).

^yAdventitious shoots.

^zNS, *, ** Nonsignificant or significant at $P \leq 0.05$ or ≤ 0.01 , respectively.

^wr = correlation coefficient of regression analysis.

MSI = modified Murashige and Skoog.

Table 3. Effects of leaf region on shoot regeneration of *A. americana* cultured on MSI medium.^z

NAA + BA ^y (μ M)	Leaf region	Callus response (%)	AdS ^x (%) after 90 d	AdS ^x number per explant after 120 d
2.68 + 13.32	Base	52.0 $\pm$ 2.9 a	84.6 a	41.3 $\pm$ 6.5 a
	Middle	38.0 $\pm$ 2.2 b	70.6 b	24.0 $\pm$ 1.7 b
	Apex	25.5 $\pm$ 0.9 c	50.0 c	5.0 $\pm$ 1.1 c
2.68 + 4.44	Base	100.0 $\pm$ 0.0 a	72.2 a	37.5 $\pm$ 2.1 a
	Middle	84.0 $\pm$ 1.7 b	50.0 b	22.8 $\pm$ 1.6 b
	Apex	56.3 $\pm$ 1.8 c	34.6 c	14.8 $\pm$ 1.3 c

^zMeans within a single column followed by different letters within the three regions represent significant difference ($P < 0.01$) by Duncan's multiple range test.

^yNAA = α -naphthaleneacetic acid, an auxin; BA = 6-benzylaminopurine, a cytokinin.

^xAdventitious shoots.

MSI = modified Murashige and Skoog.

Table 4. Effect of basal culture medium with 2.68 μ M α -naphthaleneacetic acid (NAA) and 13.32 μ M 6-benzylaminopurine (BA) on *A. americana* shoot formation.^z

Basal medium ^y	Callus response (%)	AdS ^x (%) after 90 d	AdS ^x number per explant after 120 d
MS	50.0 $\pm$ 4.4 b	43.8 $\pm$ 3.6b	15.8 $\pm$ 0.8b
MSI	64.6 $\pm$ 2.1 a	68.8 $\pm$ 3.6a	23.4 $\pm$ 2.7a
DKW	31.7 $\pm$ 1.7b	14.3 $\pm$ 2.2c	8.0 $\pm$ 0.8c
WPM	18.3 $\pm$ 2.9c	0.0d	0.0d

^zMeans within a single column followed by different letters within the same column represent significant difference ($P \leq 0.01$) by Duncan's multiple range test.

^yMSI = modified Murashige and Skoog medium; MS = Murashige and Skoog medium (Murashige and Skoog, 1962); DKW = Driver and Kuniyuki Walnut (Driver and Kuniyuki, 1984); WPM = Woody plant medium (Lloyd and McCown, 1981).

^xAdventitious shoots.

roots per shoot were significantly affected by the type and concentration of auxins (Table 5).

Analysis of variance showed significant effects of IBA, NAA, and IBA $\times$ NAA interaction on percentage of root formation and roots number (after culture 20 d) ($P < 0.01$ or $P < 0.05$). There was a positive linear correlation between IBA concentration and shoots forming percent and roots number per shoot ($P < 0.01$ or $P < 0.05$). There was a negative linear correlation between NAA and root formation (%) after culture 20 d ($r = -0.314$, $P < 0.05$) (Table 5) by regression analysis. It showed IBA was the main effect on shoot rooting in *A. americana* (positive linear correlation).

Maximum frequency of rooting (90%) and number of roots (3.4) were both obtained on medium with 4.92 μ M IBA after 20 d ($P < 0.05$) (Fig. 4A). The highest percentage of rooting (92%) occurred on medium with 1.48 μ M IBA and 1.61 μ M NAA and the highest number of roots (5.2) happened on a medium with 4.92 μ M IBA plus 5.34 μ M NAA (Table 5; Fig. 4B). However, in media supplemented with NAA alone, the rooting frequency and mean number of roots decreased as NAA concentration increased from 0.54 to 5.34 μ M (negative linear correlation) (Table 5; Fig. 4B). The media with NAA alone induced very low

Fig. 3. Histological observations of shoots (organogenesis) developed from leaf segment explant of *A. americana* induced and proliferated in MSI medium supplemented with NAA + BA at 2.68 + 13.32 (μM + μM). (A) A development shoot derived from the leaf segment explant consists of shoot apical meristem (SAM), leaf primordium (LP), and sheath leaves (SL) and connected with vascular tissue (VS) (bar = 0.1 mm); (B) a developed and mature shoot (bar = 0.2 mm). MSI = modified Murashige and Skoog medium; NAA = α -naphthaleneacetic acid; BA = 6-benzylaminopurine.

Table 5. Effect of indole-3-butyric acid (IBA) and α -naphthaleneacetic acid (NAA) concentrations on root induction by *A. americana* shoots.^z

NAA (μM)	IBA (μM)	Root (%)		Root number per shoot	
		Culture 10 d	Culture 20 d	Culture 10 d	Culture 20 d
0	0	0 f	10 e	0.0 $\pm$ 0.0 b	0.2 $\pm$ 0.1 c
0	0.49	20 d	60 d	0.2 $\pm$ 0.1 b	1.5 $\pm$ 0.6 bc
0	1.48	30 c	80 bc	0.2 $\pm$ 0.1 b	1.8 $\pm$ 0.5 bc
0	2.46	36 c	81 b	0.4 $\pm$ 0.2 b	2.6 $\pm$ 0.5 abc
0	4.92	50 b	90 ab	0.5 $\pm$ 0.2 ab	3.4 $\pm$ 0.7 ab
0.54	0	20 d	80 bc	0.2 $\pm$ 0.2 b	2.3 $\pm$ 0.7 bc
1.61	0	10 e	20 e	0.1 $\pm$ 0.1 b	0.5 $\pm$ 0.3 c
2.68	0	10 e	10 e	0.1 $\pm$ 0.1 b	0.1 $\pm$ 0.1 c
5.36	0	10 e	10 e	0.1 $\pm$ 0.1 b	0.2 $\pm$ 0.2 c
1.61	1.48	58 a	92 a	0.6 $\pm$ 0.3 ab	5.0 $\pm$ 1.1 a
2.68	2.46	50 b	80 bc	0.7 $\pm$ 0.3 ab	4.0 $\pm$ 1.6 ab
5.36	4.92	50 b	70 c	0.9 $\pm$ 0.3 a	5.2 $\pm$ 1.6 a
Analysis of variance ^y					
P value	IBA	0.0000**	0.0000 **	0.0604 NS	0.0130 *
	NAA	0.0000**	0.0000**	0.6928 NS	0.1407 NS
	IBA $\times$ NAA	0.0000**	0.0020 **	0.9683 NS	0.0493*
Regression analysis ^y					
NAA-linear	P value	0.3311 NS	0.0311*	0.1536 NS	0.1538 NS
	r ^x	0.0754	-0.314	0.0965	0.0964
IBA-linear	P value	0.0000**	0.0000**	0.0014**	0.0450*
	r ^x	0.7931	0.623	0.2775	0.1596

^zMeans within a single column followed by different letters within the same column represent significant differences ($P \leq 0.05$) by Duncan's multiple range test. Correlation of IBA, NAA, and IBA $\times$ NAA interaction to root %, no. of roots/explant was performed by bivariate analysis and linear regression in SPSS 17.0 (SPSS Inc., Chicago, IL).

^yNS, *, ** Nonsignificant or significant at $P \leq 0.05$ or ≤ 0.01 , respectively.

^xr = correlation coefficient of regression analysis.

frequencies of rooting (10% to 20%, expect 0.54 μM NAA, 80%). In addition, callus formed at the shoot bases, indicating that high NAA concentrations inhibited shoot rooting of *A. americana* (Table 5; Fig. 4B). The roots formed from media with 1.48 μM IBA plus 1.61 μM NAA (92% rooting) were short and sturdy, and callus grew at their

shoot base (Fig. 4B), whereas shoots transferred to media with IBA alone produced long, thin, and delicate roots and no calli. Thus, the medium with 4.92 μM IBA alone was considered as the best for inducing roots on *A. americana* excised shoots; it produced a high percent of rooting and healthy and the longest roots (3.5 cm) (Figs. 4A and 5).

Plantlet acclimatization. Plantlets from micropropagation transferred to ex vitro conditions and successfully acclimatized to greenhouse conditions, with an 87% survival rate after 40 d (Fig. 4C–D). The regenerated plantlets appeared morphologically normal.

Discussion

Agave americana is a potential bioenergy crop resulting from its large cellulosic biomass with very low lignin (Debnath et al., 2010). Because of its long breeding cycle, we aimed to develop micropropagation and regeneration systems that would be useful in applying in vitro technologies for its improvement such as in vitro mutations and genetic engineering. We achieved rapid micropropagation through culturing basal stems of *A. americana*, similar to previously reported systems for *A. fourcroyde* (Robert et al., 1987), *A. tequilana* (Valenzuela-Sánchez et al., 2006), and *A. parrasana* (Santacruz-Ruvalcaba et al., 1999). Micropropagated shoots can be readily rooted either in vitro or ex vitro, and acclimatized to greenhouse conditions.

The most important factors in successfully micropropagating *A. americana* were cytokinin and auxin levels. Cytokinin in the medium was reported to be an essential factor for micropropagating *Agave* species. Das (1992) and Hazra et al. (2002) both found the highest rates of *A. sisalana* regeneration were obtained using BA as the only PGR. Our results support previous results with *Agave* species because shoots from basal stems on BA-only medium supported proliferation and produced healthy shoots. We also found that shoot proliferation, leaf length, and FW gain of *A. americana* were influenced by both nutrient salt composition and BA concentration. Low BA and high nutrient salt levels or high BA and low nutrient salt levels were beneficial to leaf elongation.

The exogenous PGR levels, formulation of the basal salts in the medium, and physiological status of the explants can be key factors in shoot induction using leaf as the explant. Leaf segments (*A. sisalana*) did not develop a callus structure or shoots (*A. sisalana*) supplemented with BA alone or combination with 2,4-dichlorophenoxyacetic acid (2,4-D) in medium (Das, 1992; Valenzuela-Sánchez et al., 2006). Shoot regeneration of *A. sisalana* was obtained from compact green callus of immature leaf explants containing BA alone (Hazra et al., 2002). However, leaves of *A. tequilana* (Portillo et al., 2007) and *A. salmiana* (Flores-Benitez et al., 2007) achieved somatic embryos on MS medium with BA and 2,4-D or BA and NAA.

In our study, the proper concentrations of NAA and BA combination were the most critical factor for inducing shoot formation from leaves. The presence of NAA in the medium was essential for shoot formation (positive linear correlation), but the BA concentration determined the number and formation of shoots. Furthermore, *A. americana* developed shoots through de novo organogenesis, probably because NAA was used

Fig. 4. Effect of IBA and NAA on inducing rooting from shoots and plantlets in ex vitro condition. (A) Rooting from shoots at different concentration of IBA; (B) shoots rooting at different concentration of NAA and IBA combination NAA; (C) plantlets in ex vitro condition; (D) shoot rooting in an ex-vitro condition. IBA = indole-3-butyric acid; NAA = α -naphthaleneacetic acid.

Fig. 5. Effects of different concentrations of indole-3-butyric acid (IBA) and α -naphthaleneacetic acid (NAA) on the length of the longest root on *Agave americana* shoots after 20 d of culture. Means followed by different letters above the bars indicate significant differences ($P < 0.01$) among treatments according to the Duncan's multiple range test.

instead of 2,4-D. 2,4-D has been shown to be highly potent in inducing somatic embryogenesis, whereas NAA has been used to induce shoot organogenesis when used in combination with cytokinin (Annapurna and Rathore, 2010; Nguyen et al., 2013).

Agave plants have long leaves and leaf length and maturity increases as the plant ages, potentially affecting organogenesis in different leaf regions. Our results showed that the regeneration capacity decreases from the basal end toward to the apical end of the leaf. This result was similar to those by Garcia-Luis et al. (2006), Germanà et al. (2008), and Marques et al. (2011), who reported that the best results were obtained using basal segments or regions nearest to the cotyledons.

Goh et al. (1995) and Moreira-Dias et al. (2001) also reported that the epicotyl axis near the base and cotyledons had maximal organogenic potential in *Citrus grandis* Osbeck and Troyer citrange. Because *Agave* is a monocotyledonous species, the distal end of the leaves is more mature and, therefore, less responsive to hormones.

Nutrients and vitamins in culture media strongly affect the success of in vitro tissue culture (Rounsaville et al., 2011; Ruzic et al., 2004). The $\text{NO}_3^-/\text{NH}_4^+$ balance in the medium was a key factor controlling callus growth and organogenesis of *A. sisalana* (Robert et al., 1987). Valenzuela-Sánchez et al. (2006) used MS medium without glycine, nicotinic acid, or pyridoxine and with 9.4 mM KNO_3 and 10.3

mM NH_4NO_3 to induce callus and regenerate plants. In our study, high-salt media (MSI and MS) were beneficial to shoot induction of *A. americana*; lower-salt media such as DKW or WPM were ineffective. Most *Agave* tissue culture studies have used MS medium (high salt) (Hazra et al., 2002; Martínez-Palacios et al., 2003; Portillo et al., 2012). Thus, a high-salt medium is more favorable for *Agave* shoot induction. In addition, the vitamin composition is important.

Successful rooting from microcuttings usually depends on the plant species and auxins used. NAA, IBA, and indole-3-acetic acid (IAA) are usually added to media to promote adventitious rooting of cultured shoots. However, shoots of *A. tequilana* (Valenzuela-Sánchez et al., 2006), *A. arizonica* (Powers and Backhaus, 1989), and *A. sisalana* (Das, 1992) rooted in PGR-free medium. However, shoots of *A. fourcroydes* (Robert et al., 1987, 2006) and *A. sisalana* (Hazra et al., 2002) required both 2,4-D and IAA for rooting to occur. In our study, the rooting rate of *A. americana* was very low (10%) in PGR-free medium, but medium with IBA alone or in combination with NAA yielded a high rooting frequency (greater than 90%) of individual shoots and number of roots, whereas NAA alone at high concentrations inhibited rooting. Similar results were reported for *A. attenuate* (Li et al., 2003), *Rosa kordesii* (Arnold et al., 1995), and *Camptotheca acuminata* (Chen et al., 2004), suggesting that plant species require different auxins and concentrations to stimulate adventitious rooting.

In conclusion, we have developed a shoot propagation protocol for *A. americana* using basal stems. The optimum medium for proliferation is MSI contains 13.32 μM BA. This protocol can be used to mass production for experimental materials and elite clones from a breeding program in vitro manipulation. We also developed a protocol for plant generation through shoot formation from leaf explants through organogenesis. The optimum protocol uses basal sections of leaf tissues and culture on MSI media containing 2.68 μM NAA and 13.32 μM BA. The optimum medium for shoots rooting is 1/2 MSI containing 4.92 μM IBA alone. This is the first complete protocol for the micropropagation and plant regeneration of *A. americana*. The plant regeneration protocol is a prerequisite for many in vitro manipulation technologies such as in vitro mutation, selection, and genetic engineering.

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