Gayana Bot. 74(1): 120-130, 2017 ISSN 0016-5301
Artículo original

Baybean (*Canavalia rosea* (Sw.) DC.); organogenesis, morphological and anatomical studies

Estudio de organogénesis, morfología y anatomía en el Baybean (*Canavalia rosea* (Sw.) DC.)

Sadegh Mohajer*[1,2], Rosna Mat Taha[1], Normadiha Mohamed[1] & Ummi Nur Abdul Razak[1]

[1]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2] Department of Molecular Biotechnology and Bioinformatics, Faculty of Science, Prince of Songkla University, Songkla, 90112, Thailand
*mohajer.ae@gmail.com

ABSTRACT

Morphological and anatomical studies comprised leaf venation, histological analysis and epidermal peeling were carried out on *Canavalia rosea* (Sw.) DC.). Anatomical studies of the leaf and root determined the presence of cuticle and oil glands, arrangement of cells and the structure of vascular system. The dorsiventral leaf with pinnate venation and amphistomatic leaf with paracytic type stomata were embedded below the epidermis. Trichomes and oil glands were also observed on both adaxial and abaxial surfaces of leaf. Longitudinal sections of the callus showed the existence of meristematic cells, which could give rise to plant regeneration. In the process of organogenesis, adventitious shoots emerged from leaf and stem explants within 2 months after culturing on MS media fortified with 6-Benzylaminopurine (1.0-2.0 mg/l). Ultimately, *in vitro* germination was successfully established for the propagation and future conservation programs of this species.

Keywords: Leaf architecture, histology, hormone, 6-benzyladenine, trichomes, SEM.

RESUMEN

Se realizaron estudios morfológicos y anatómicos de venación foliar, análisis histológico y peeling epidérmico en *Canavalia rosea* (Sw.) DC). Los estudios anatómicos de la hoja y de la raíz determinaron la presencia de la cutícula y de las glándulas de aceite, la disposición de células y la estructura del sistema vascular. La hoja dorsiventral con venación pinnada y la hoja anfistomática con estomas paracíticos estaban incrustados debajo de la epidermis. También se observaron tricomas y glándulas oleosas en las superficies adaxial y abaxial de la hoja. Las secciones longitudinales del callo mostraron la existencia de células meristemáticas que podrían dar lugar a la regeneración de las plantas. En el proceso de organogénesis, brotes adventicios surgieron de los explantes de hoja y tallo en los 2 meses después del cultivo en medios de MS fortificados con 6-bencilaminopurina (1,0-2,0 mg / l). En última instancia, la germinación in vitro se estableció con éxito para la propagación y los futuros programas de conservación de esta especie.

Palabras clave: Arquitectura foliar, histología, 6-benciladenina, hormona, tricomas, SEM.

INTRODUCTION

Canavalia rosea (Sw.) DC. (Fabaceae) is a species of flowering plants in the family Leguminosae. It is well known as beach bean, baybean, seaside jack-bean and coastal jack bean (Vatanparast 2010). *Canavalia rosea* is distributed throughout coastal areas of the subtropics and tropics around the world. Baybean is one of the most common and widespread tropical seacoast plants, trailing on beaches at the outer limits of land vegetation (Padmavathy 2010).

This species is economically important and can be used as food, fodder, fertilizer, a source of bioactive compounds and pharmaceutical substances (Sridhar & Bhagya 2007). Jayavardhan *et al.* (2008) reported that *Canavalia rosea* has valuable phytochemicals and toxins that are well known for medicinal purposes. Moreover, this species has a notable effect on the stability of soil.

A main part of the genetic engineering process of plants is to place a new genetic information into the plant cells with regeneration ability (Colby *et al.* 1991a). Histological studies can aid in understanding the regeneration process in studies of plant transformation (Colby *et al.* 1991b).

In addition, Pathan *et al.* (2008) reported that plant surfaces could be viewed at high resolution using Scanning Electron Microscope (SEM). Wide varieties of plant microstructures, including light reflection and water absorption structures, have been defined by the SEM. Most significant threat in plant life can be referred to high temperature, because of intense radiation and increment of water loss as a growth limiting factor (Mohajer *et al.* 2014).

Micropropagation offers the possibility of large scale multiplication of plant materials. Simulation of action potential propagation in plants depends on different factors such as explant, plant regulator growth and ambient condition. (Gloria *et al.* 2008). Micropropagation of *Canavalia rosea* (Sw.) DC. has been carried out for the mass propagation of the species (Cunha & Sridhar 2010). Also, tissue culture technique may improve the plant productivity by developing the disease resistance and yield enhancement (Khan *et al.* 2009).

The present study was designed; to investigate the macro- and micro-morphological characteristics, anatomical and histological properties of *in vivo* and *in vitro* grown plants, and to describe the procedure for the establishment of different types of callus and organogenesis from various explants of *Canavalia rosea* by using different concentrations of BAP and NAA.

MATERIALS AND METHODS

Explants source

Seeds of *Canavalia rosea* were collected from coastal region of Port Dickson, Negeri Sembilan, Malaysia. The seeds were washed thoroughly under running tap water for one hour, followed by treatment with two drops of Tween 20 for 15 min and washed three to five times with distilled water. Seeds were then surface sterilized with different concentrations (70%, 50%, 30%, 20%, and 10%) of sodium hypochlorite solution (Clorox) for 5-7 min each and rinsed thoroughly with sterile distilled water. Finally, the seeds were treated with 70% ethanol for 1 min and rinsed with sterile distilled water all over again in the laminar air flow cabinet. Seeds were cultured aseptically on MS basal medium supplemented with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. The pH of medium was adjusted to 5.8 by using NaOH and HCl. The media were autoclaved at 121 ºC for 20 min. The aseptic seedlings germinated from sterilized seeds were used as explant sources.

Organogenesis and callus induction

Juvenile leaf and stem sections of each explant (5 mm in length) were placed on MS medium supplemented with various concentrations of BAP (0.5-2.0 mg/l) and NAA (0.5-2.0 mg/l). Calli obtained from the stem and leaf explants were sub-cultured at a regular interval of 1 month on the same media. All cultures were maintained in culture room at 25 ± 1 ºC under 16 h light and 8 h dark cycle.

Morphological studies

Morphological studies involved macro-morphology and micro-morphology of *Canavalia rosea* from *in vivo* and *in vitro* grown plants. Macro-morphology studies consisted of the vegetative and reproductive characteristics of the specimens, viewed under a binocular microscope. Micro-morphology studies involved the leaf venation of *Canavalia rosea* viewed under Scanning Electron Microscope (Philips SEM 515, Netherlands). For leaf venation, the fresh leaves were soaked overnight in 30% NaOH solution. Subsequently, the leaves were immersed in 15% NaOH solution. The solutions were replaced daily until the leaf became clear. The leaves were then washed using distilled water for 3 times. The upper and lower laminae of leaves were scrapped off using forceps. Dehydration and staining of the leaves were done using different serial of dilution (30% ethanol for 15 min, 40% ethanol for 15 min, Safranin 'O' for 2 min, 70% ethanol for 5 min, 95% ethanol for 5 min, absolute ethanol for 5 min, 50% xylene: 50% ethanol for 5 min and 100% xylene for 5 min) (Mackay *et al.* 1998).

The abaxial and adaxial surfaces of leaves such as trichome and stomata were also observed under SEM. The samples were fixed in 4% glutaraldehyde in cacodylate buffer for at least 4 h. The samples were then washed twice with cacodylate buffer for 10 min each and fixed in 1% Osmium for 1 h. After that, the samples were rinsed twice with distilled water for 10 min each. The samples were then dehydrated in a graded ethanol series (30% - 100%) for 15 min each. Following that, the samples were dehydrated in a series of ethanol and acetone mixtures (with ethanol to an acetone ratio of 3:1, then 1:1 and 1:3) for 15 min each. The samples were then dehydrated in pure acetone three times for 20 min and exposed to critical point drying (CPD) process for 1 h. Then, the sample was mounted on stubs using double sided tape and coated with gold cover using a sputter coater (Mohajer *et al.* 2014).

Anatomical studies

Epidermal peeling - The leaves were soaked in 10% Nitric Acid overnight. The leaves were then washed using distilled water. Sharp forceps were used to suction out the abaxial and adaxial surfaces of leaf. Each leaf was immersed in 5% Clorox for 5-10 min. The leaves were rinsed using distilled water again. Subsequently, the samples were immersed in Safranin 'O' for 10 min. Then, a series of gradations of ethanol dilution (30%, 50%, 75%, 90%, 95%, and 100%) were used to dehydrate and stain the leaf each for 2 min. After that, the leaves were immersed in ethanol: xylene in

solutions of 1:1 for 2 min. Finally, the samples were kept in xylene for 2 min. Canada Balsam was used to fix the specimens on the slides. The measurement of stomatal index of the leaves was also made (Ogunkunle & Oladele 2008).

Histological analysis - *In vitro* and *in vivo* leaves and roots were fixed in FAA solution containing 90 ml ethyl alcohol (70%), 5 ml of glacial acetic acid and 5 ml of formaldehyde for 48 h (Wahua *et al.* 2014). The samples were washed with 70% alcohol for a few times and followed by dehydration in various combinations of TBA (tertiary butyl alcohol), 95% of ethyl alcohol, 100% of ethyl alcohol and just distilled water, respectively. Then, the samples were infiltrated in a mixture of paraffin oil and TBA and kept overnight. The samples were then transferred into melted 49 °C paraffin wax for an overnight, thus transferred into 56 °C melted wax and kept for 24 h. The paraffin wax was poured into the paper boat for embedding process. The samples were stained with Safranin 'O' and Alcian Blue and mounted on the slide by using Albumin. The slides were viewed under an Axioskop Zeiss (Germany) microscope attached to AxioCam MRc video camera and were then analyzed using AxioVision 4.7 software.

Data analysis

The ANOVA was performed for each experiment and means were compared using Duncan's multiple range tests ($p<0.05$) through SAS (9.2) software. All data for each test are the average of 30 replications for comparison of values and were recorded as the mean ± standard error (SE).

RESULTS

Organogenesis studies

Direct organogenesis of *Canavalia rosea* was achieved when leaf and stem explants were cultured on MS media supplemented with various concentrations of BAP and NAA. Stem and leaf explants gave the highest shoot formation with 43.75% and 37.50%, respectively, on MS medium supplemented with 2.0 mg/l BAP (Table 1, Fig. 1). Multiple shoots were regenerated after more than 2 months of culture on MS medium fortified with 2.0 mg/l BAP (Fig. 1). The direct root formation was achieved in stem and leaf explants cultured on MS supplemented with NAA (0.5-3.0 mg/l). The results verified that MS medium with the addition of 1.5 mg/l NAA was the optimum medium for adventitious root formation from stem explant (Table 2).

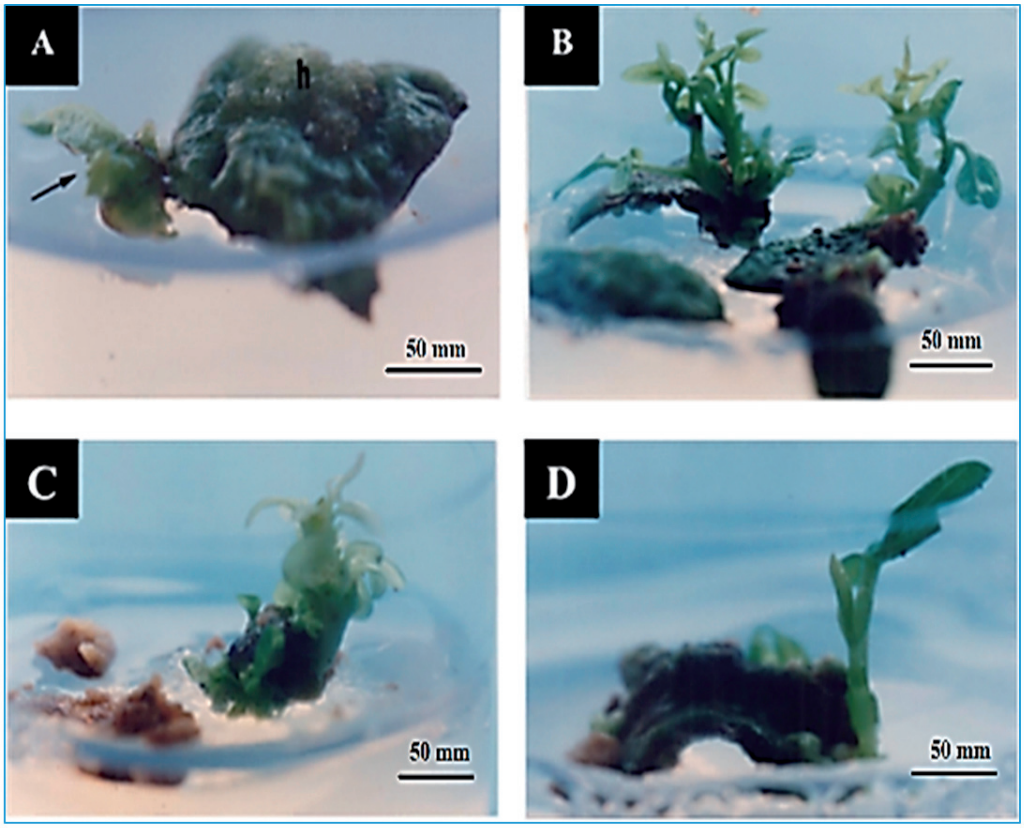

Figure 1. Formation of shoot in leaf explants on MS medium supplemented with (A) 1.0 mg/l BAP, (B) 2.0 mg/l BAP, (C) 3.0 mg/l BAP and (D) 4.0 mg/l BAP. / Organogénesis directa desde explantes foliares de *Canavalia rosea* cultivadas en medio MS. A) 1.0 mg/l BAP; B) 2.0 mg/l BAP; C) 3.0 mg/l BAP; D) 4.0 mg/l BAP.

Table 1. Effect of different concentrations of BAP (6-Benzylaminopurine) on leaf and stem explants of *Canavalia rosea* grown on MS media. Mean ± SE, n=30. / Efecto de diferentes concentraciones de BAP (6-Benzylaminopurine) en la formación de explantes de brotes y hojas de *Canavalia rosea* cultivadas en medio MS. Promedio ± Desviación Estandar, n=30.

BAP hormone (mg/l)	Explant	No. of shoot	Regeneration (%)	Fresh weight callus (g)	Observations
1	Leaf	2	22.23 ± 0.5^{c}	0.06 ± 0.01^{c}	Green callus formed after 5 days
	Stem	1	25.00± 0.7^{c}	0.02 ± 0.01^{d}	Green callus formed after 5 days
2	Leaf	6	37.50± 0.4^{b}	0.25 ± 0.02^{b}	Green callus formed after 5 days
	Stem	3	43.75± 0.4^{a}	0.09 ± 0.01^{c}	Green callus formed after 5 days
3	Leaf	4	33.33± 0.5^{b}	0.26 ± 0.03^{b}	Green callus formed after 5 days
	Stem	1	38.89± 0.5^{b}	0.08 ± 0.02^{c}	Green callus formed after 5 days
4	Leaf	4	14.29± 0.3^{d}	0.25 ± 0.05^{b}	Green callus formed after 5 days
	Stem	1	25.00± 0.6^{c}	0.09 ± 0.00^{c}	Green callus formed after 5 days
5	Leaf	0	0	0.35 ± 0.06^{a}	Green callus formed after 5 days
	Stem	0	0	0.12 ± 0.03^{c}	Green callus formed after 5 days
6	Leaf	0	0	0.09 ± 0.01^{c}	Green callus formed after 5 days
	Stem	0	0	0.03 ± 0.00^{d}	Green callus formed after 5 days

Same letters indicate significantly different as per Duncan's multi-range test at p≤0.05. / Letras distintas indican diferencias significativas según Duncan's multi-range test a p≤0,05.

Table 2. Effect of different concentrations of NAA (1-Naphthaleneacetic acid) on leaf and stem explants of *Canavalia rosea* grown on MS media. Mean ± SE, n=30. / Efecto de diferentes concentraciones de NAA (1-Naphthaleneacetic acid) en la formación de explantes de brotes y hojas de *Canavalia rosea* cultivadas en medio MS. Promedio ± Desviación Estandar, n=30.

NAA Hormone (mg/l)	Explant	No. of root	Fresh weight callus (g)	Observations
0.5	Leaf	2	0.09 ± 0.01^{c}	White callus formed after 7 days
	Stem	1	0.05 ± 0.01^{c}	White callus formed after 4 days
1.0	Leaf	2	0.22 ± 0.01^{b}	White callus formed after 6 days
	Stem	2	0.09 ±0.01^{c}	White callus formed after 4 days
1.5	Leaf	1	0.18 ± 0.02^{b}	White callus formed after 5 days
	Stem	4	0.05 ± 0.02^{c}	White callus formed after 4 days
2.0	Leaf	1	0.32 ± 0.02^{a}	White callus formed after 5 days
	Stem	4	0.14 ± 0.01^{bc}	White callus formed after 5 days
2.5	Leaf	1	0.41 ± 0.01^{a}	White callus formed after 5 days
	Stem	3	0.18 ± 0.03^{b}	White callus formed after 5 days
3.0	Leaf	1	0.38 ±0.01^{a}	White callus formed after 5 days
	Stem	2	0.13 ±0.01^{bc}	White callus formed after 5 days

Same letters indicate significantly different as per Duncan's multi-range test at p≤0.05. / Letras distintas indican diferencias significativas según Duncan's multi-range test a p≤0,05.

Different friable callus from stem and leaf explants formed after a week of culture on different combinations of MS supplemented with 0.5-2.0 mg/l BAP and 0.5-2.0 mg/l NAA. The highest fresh callus weight was obtained in leaf explants on MS media supplemented with 0.5 mg/l BAP and 2.0 mg/l NAA (1.36±0.07g). Also in presence of 1.0 mg/l BAP and 1.5 mg/l NAA, a remarkable increase in average fresh weight of white and yellow callus was obtained from stem explants with 1.21 g (Table 3, Fig. 3a).

Macro-morphological characteristics

It was observed that *Canavalia rosea* has hairy stem and leaf. The leaf has compound with three fleshy and ellipse leaflets (Fig. 3). It means that the leaf morphology of *Canavalia rosea* was 3-leaflet composite. Leaf tip shape was emarginate, meanwhile the leaf base shape was cuneate. The *in vitro* leaf also showed the same characteristics (Fig. 3c). The major difference between *in vivo* and *in vitro* leaves was observed in the size due to the limited space in *in vitro* growth. The flowers were purplish pink, complete and bisexual. The *in vitro* flowers bloomed in summer, while the flowering occurred 2 months later in *in vivo* growth culture. Pods were oblong, inert and dark brown in color. The roots were fibrous with nodules due to the association with the soil bacteria in *in vivo* culture (Fig. 4).

Micro-morphological characteristics

In leaf venation study, the method reported by Hickey (1979) was followed. The leaf venation of *Canavalia rosea* was bipinnate and venation size was large. The deflection angle of secondary veins was more than 80°. Microscopic studies of the structures of *in vivo* and *in vitro* leaves were done using Scanning Electron Microscope (SEM). It was observed that the stomata were amphistomatic on both sides, abaxial and adaxial. Stomata distributions were more dense on abaxial surface compared with adaxial surface (Fig. 5). Stomata became visible on the anticlinal wall of epidermic layer. Trichomes also appeared on both adaxial and abaxial surfaces of *in vivo* and *in vitro* grown leaves. Trichomes were unicellular and oil glands were only observed on *in vivo* grown leaves (Fig. 6).

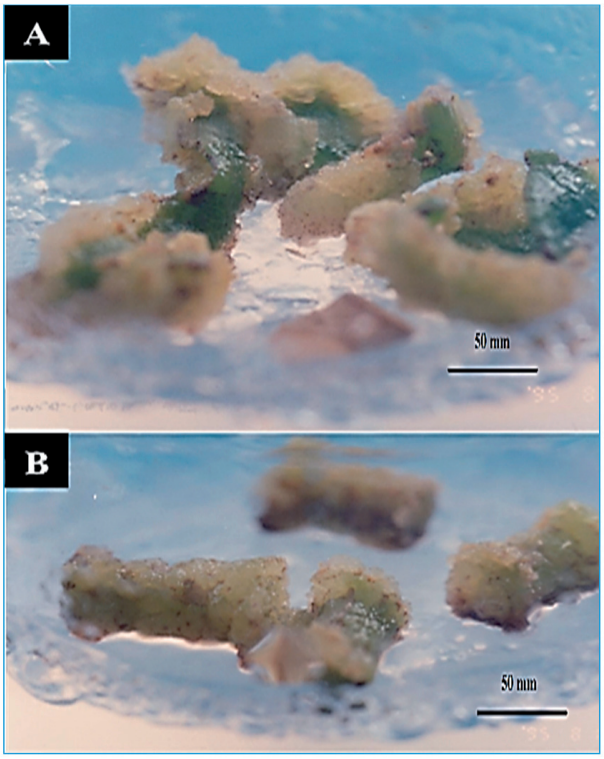

Figure 2. A) Induction of callus in leaf and B) stem explants on MS medium supplemented with 2.0 mg/l NAA and 0.5 mg/l BAP after 2 weeks cultured. / A) Callo inducido a partir de explantes foliares y B) de brotes en 2,0 mg/l NAA y 0,5 mg/l BAP medio MS durante 14 días.

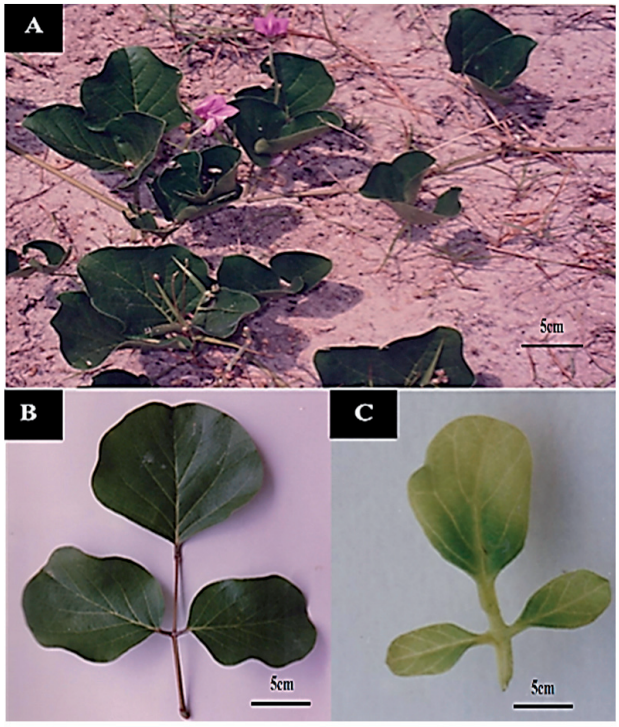

Figure 3. (A) Intact plants of *Canavalia rosea* grown trailing on the ground and form mats of foliage at the seaside of Port Dickson, Malaysia. (B) *in vivo* grown leaf and (C) *in vitro* grown leaf. / A) Plantas intactas de *Canavalia rosea* crecen arrastrándose en el suelo y forman matas en la playa de Port Dickson, Malasia. Hojas cultivadas B) *in vivo*; C) *in vitro.*

Table 3. Effect of different concentrations of NAA (1-Naphthaleneacetic acid) and BAP (6-Benzylaminopurine) on leaf and stem explants of *Canavalia rosea* grown on MS media. Mean ± SE, n=30./ Efecto de diferentes concentraciones de NAA (1-Naphthaleneacetic acid) y BAP (6-Benzylaminopurine) sobre explantes de hoja y brotes de *Canavalia rosea* cultivadas en medio MS. Promedio ± Desviación Estandar, n=30.

Hormone (mg/l)		Explant	Fresh weight callus (g)	Observations
NAA	BAP			
0.5	0.5	Leaf	0.77 ± 0.04^{b}	Green callus formed after 7 days
		Stem	0.13 ± 0.02^{e}	White and yellow callus formed after 5 days
	1.0	Leaf	0.39 ± 0.02^{d}	Green callus formed after 7 days
		Stem	0.56 ± 0.03^{c}	White and yellow callus formed after 6 days
	1.5	Leaf	0.56 ± 0.05^{c}	Green callus formed after 8 days
		Stem	0.22 ± 0.03^{e}	White and yellow callus formed after 6 days
	2.0	Leaf	0.74 ± 0.07^{b}	Green callus formed after 9 days
		Stem	0.35 ± 0.03^{d}	White and yellow callus formed after 6 days
1.0	0.5	Leaf	0.59 ± 0.03^{c}	Green callus formed after 7 days
		Stem	0.41 ± 0.04^{d}	White and yellow callus formed after 6 days
	1.0	Leaf	0.81 ± 0.05^{b}	Green callus formed after 8 days
		Stem	0.38 ± 0.01^{d}	White and yellow callus formed after 6 days
	1.5	Leaf	0.82 ± 0.09^{b}	Green callus formed after 8 days
		Stem	0.39 ± 0.05^{d}	White and yellow callus formed after 6 days
	2.0	Leaf	0.83 ± 0.07^{b}	Green callus formed after 8 days
		Stem	0.41 ± 0.05^{d}	White and yellow callus formed after 6 days
1.5	0.5	Leaf	0.64 ± 0.05^{bc}	Green callus formed after 8 days
		Stem	0.68 ± 0.03^{bc}	White and yellow callus formed after 6 days
	1.0	Leaf	0.74 ± 0.03^{b}	Green callus formed after 8 days
		Stem	1.21 ± 0.03^{a}	White and yellow callus formed after 6 days
	1.5	Leaf	0.84 ± 0.04^{b}	Green callus formed after 8 days
		Stem	0.68 ± 0.03^{bc}	White and yellow callus formed after 6 days
	2.0	Leaf	0.54 ± 0.02^{c}	Green callus formed after 8 days
		Stem	0.32 ± 0.04^{d}	White and yellow callus formed after 6 days
2.0	0.5	Leaf	1.36 ± 0.07^{a}	Green callus formed after 8 days
		Stem	0.71 ± 0.02^{b}	White and yellow callus formed after 6 days
	1.0	Leaf	0.63 ± 0.03^{bc}	Green callus formed after 8 days
		Stem	0.30 ± 0.01^{d}	White and yellow callus formed after 6 days
	1.5	Leaf	0.73 ± 0.02^{b}	Green callus formed after 8 days
		Stem	0.56 ± 0.05^{c}	White and yellow callus formed after 6 days
	2.0	Leaf	0.41 ± 0.01^{d}	Green callus formed after 8 days
		Stem	0.15 ± 0.02^{e}	White and yellow callus formed after 6 days

Same letters indicate significantly different as per Duncan's multi-range test at $p \leq 0.05$. / Letras distintas indican diferencias significativas según Duncan's multi-range test a $p \leq 0{,}05$.

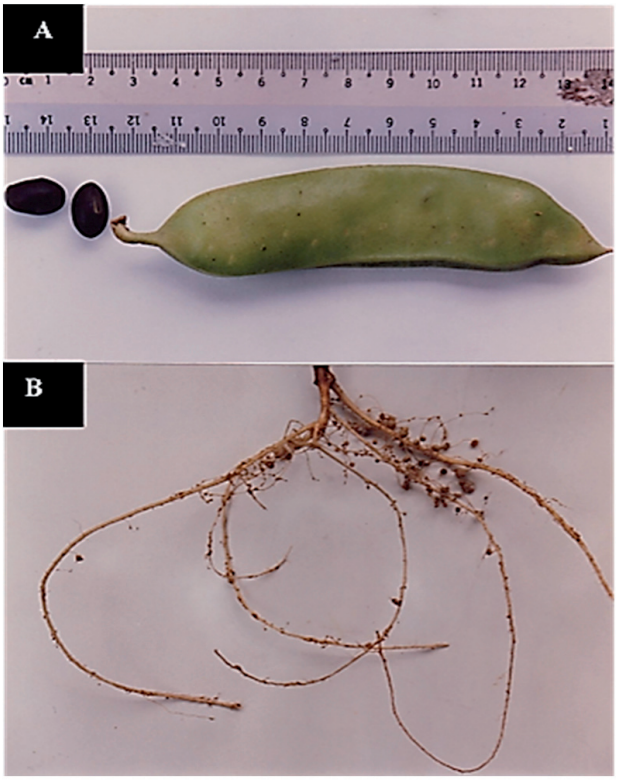

Figure 4. (A) Fruit contains black and oblong-shaped seeds. (B) Fibrous and nodular roots. / A) Fruto con semillas negras de forma oblonga; B) Raíces fibrosas y nodulares.

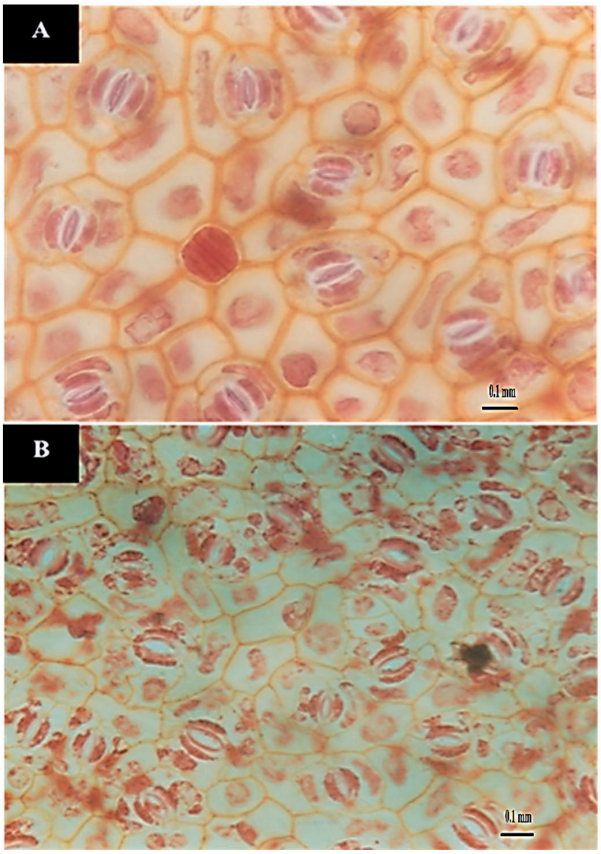

Figure 5. (A) *In vitro* adaxial surface showing the epidermis cells are not homogeneous and distributions of stomata are less dense. (B) *In vivo* abaxial surface showing the shapes and sizes of epidermis cells are not homogeneous and distributions of stomata are dense. / (A) la cara adaxial *in vitro* de la epidermis muestra que las células no son homogéneos y la distribución de los estomas es menos densa. (B) cara abaxial *in vivo* muestra que la forma y tamaño de las células epidérmicas no son homogéneas y la distribución de los estomas es densa.

Table 4. Anatomical characterisation of *in vivo* and *in vitro* leaf of *Canavalia rosea*. / Características anatómicas de hojas *in vivo* e *in vitro* de *Canavalia rosea*.

Anatomical Characteristic	*In vivo*			*In vitro*		
	Stipule	Midrib	Tip	Stipule	Midrib	Tip
Vascular system	Closed U-shaped	Closed U-shaped	Closed U-shaped	Kidney	Kidney	Slightly round
Palisade Cell	2 layers	2 layers	2 layers	1 layer	1 layer	1 layer
Cell arrangement	Less Dense	Less Dense	Less Dense	Dense	Dense	Dense
Cell shape	Round, oval, curved	Round, oval, curved	Round, oval, curved	Round, oval	Round, oval	Round, oval
Epidermis Layer	1 layer	1 layer	1 layer	1 layer	1 layer	1 layer
Cuticle Layer	visible	visible	visible	invisible	invisible	invisible
Oil Gland	8-9 Glands	5-6 Glands	1-2 Glands	invisible	invisible	invisible

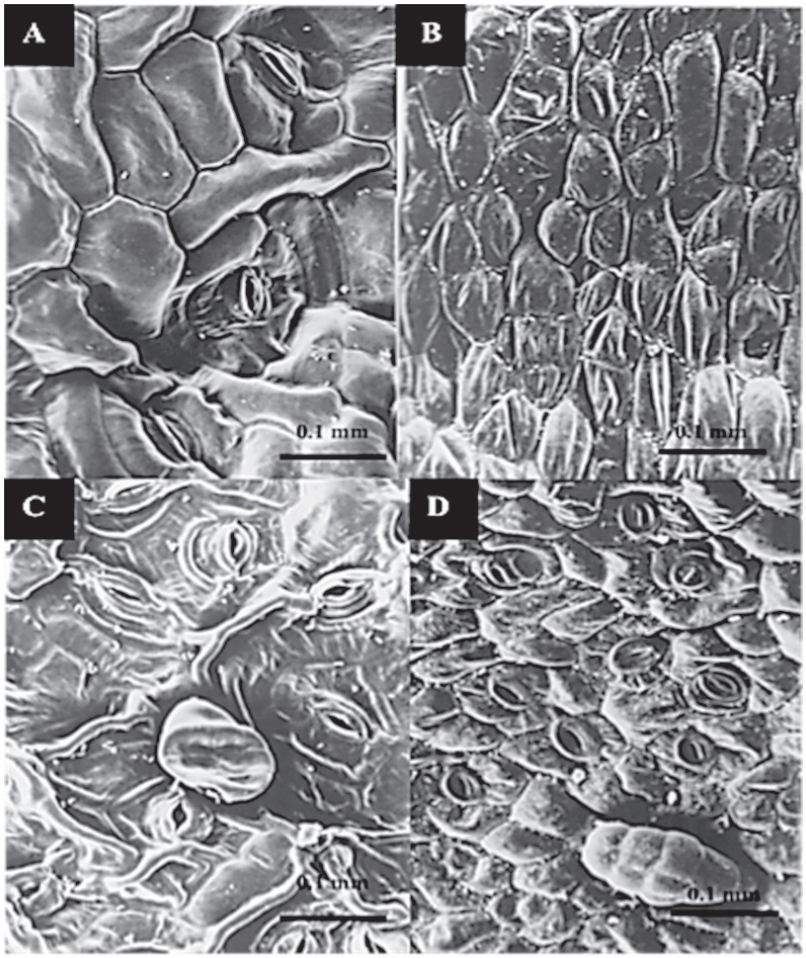

Figure 6. SEM image. The distributions of stomata on adaxial surface of *in vivo* (A) and *in vitro* (B) which are less dense compared with abaxial surface of *in vivo* (C) and *in vitro* (D). Stomata become visible on the anticlinal walls of epidermis layer. / Microfotografías electrónicas (SEM). A) Distribuciones de estomas en superficie adaxial *in vivo*; B) distribuciones de estomas en superficie adaxial *in vitro*; C) distribuciones de estomas en superficie abaxial *in vivo*; D) distribuciones de estomas en superficie abaxial *in vitro*.

Histological studies

Histological analysis has been done on stipule, midrib and tip of *in vivo* and *in vitro* leaves. Anatomical characterisation of *in vivo* and *in vitro* leaves of *Canavalia rosea* are indicated in Table 4. The *in vivo* leaf showed vascular bundle formed closed U-shaped with uniseriate epidermis on both adaxial and abaxial leaf surfaces. Oil glands were also displayed on stipule and shoot tips. Two palisade cell layers and span cells with various sizes and shapes were also observed (Fig. 7).

Vascular bundle on stipule and midrib formed kidney-shaped, while the vascular bundles on the tips were round-shaped in *in vitro* grown leaf (Table 4, Fig. 8). Cuticle layer and oil glands were invisible on *in vitro* leaf, meanwhile *in vivo* root showed 3 different type of tissues such as epidermis, cortex and vascular bundle (Fig. 9a). Root cortex showed the presence of bacteriod zone containing nitrogen fixing bacteria. Xylems were more visible than phloems on the vascular cylinder. Histological studies of the *in vitro* root showed undeveloped secondary and adventitious roots after 2 weeks (Fig. 9b).

This species had association with nitrogen fixing bacteria, that could produce root nodule structure. *In vivo* roots of *Canavalia rosea* showed succulent characteristics, whereas parenchyma cells at cortex can reserve the water to prevent dehydration. Moreover, cell layers of root cortex were reduced in *in vitro* growth and vascular cylinder was enlarged compared with *in vivo* root, which reduce the distance of vascular cylinder to absorb more water and nutrients.

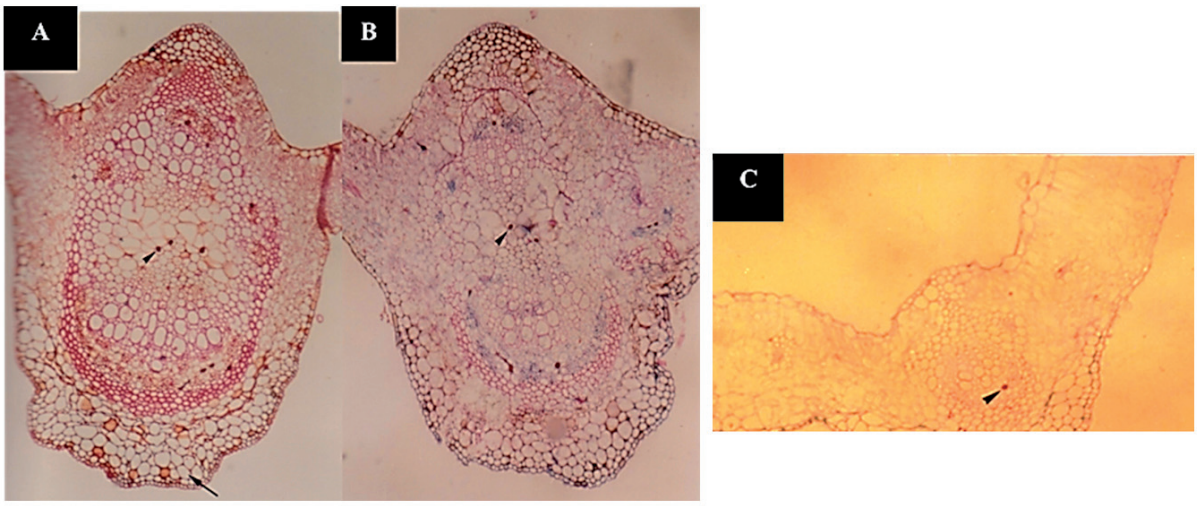

Figure 7. Histology of *in vivo* leaf at (A) stipule (B) midrib and (C) tip parts. Orientation of collateral vascular bundle formed closed U-shaped. There are several oil glands (➧) in the cells. The arrow shows the parenchyma cells act as water reservoir in the leaf. (40x). / Análisis histológico de la hoja *in vivo* A) Estípula; B) Nervadura; C) Punta. Varias glándulas sebáceas (➧) en las células. Flecha muestra que las células del parénquima actuan como reservorio de agua en la hoja. (40x).

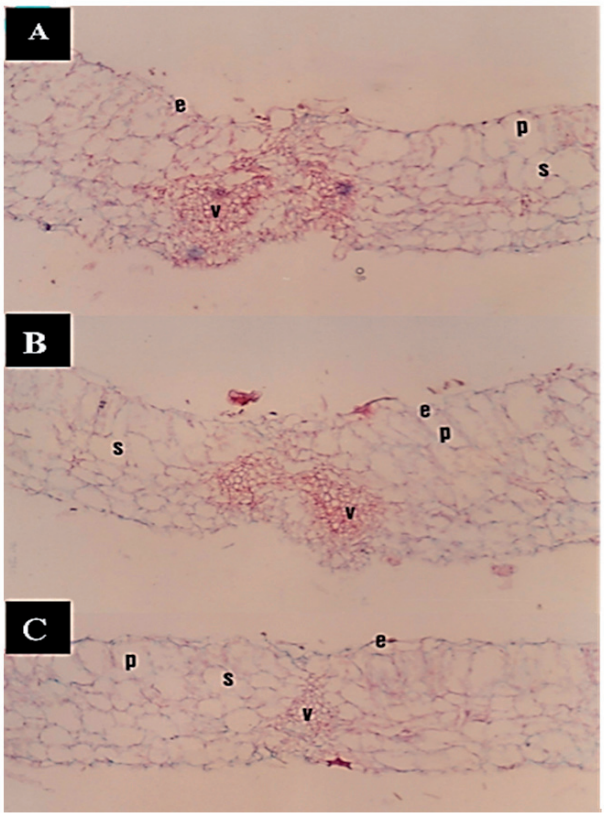

Figure 8. Histology of *in vitro* leaf shows vascular bundle (v) in kidney-shaped, epidermis (c) palisade parenchyma cells (p) spongy mesophyll cell (s) in leaf stipule (A), midrib (B) and tip (C). (40x). / Análisis histológico de *in vitro* de la hoja con haz vascular. v, epidermis; c, células del parénquima; p, célula del mesófilo; s, estípula. A) Estípula; B) nervadura; C) punta. (40x).

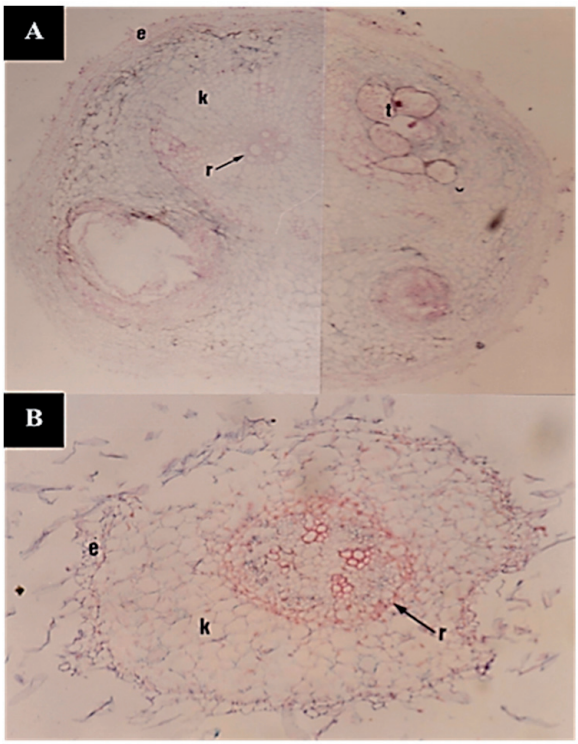

Figure 9. Histology of roots shows three tissue systems which are epidermis (e), cortex (k), and vascular cylinder (r). (A) *In vivo* root with zone of bacteriod (t), (B) *In vitro* root without zone of bacteriod. (40x). / Análisis histológico de raíces con three sistemas de tejidos, e, epidermis; k, corteza; r, cilindro vascular. A) Raíces *in vivo* con zona de bacteriode (t); B) Raíces *in vitro* sin zona de bacteriode (40x).

DISCUSSION

Pawan & Rana (2003) reported that *in vitro* culture and advanced plant breeding help to improve the quality of legumes. Cunha & Sridhar (2010) also reported that propagation of *Canavalia* spp. through seeds using conventional methods is not reliable. In this respect, various factors were studied to establish a remarkable *in vitro* regeneration protocol in this species.

Types of explants, effect of hormones and physical factors were assessed in order to obtain an optimum regeneration system for *Canavalia rosea.* MS medium supplemented with 2.0 mg/l BAP, was optimal for regeneration of *Clitoria ternatea* as reported by Mohamed and Taha (2011). The stimulating effect of BAP on multiple shoots formation has been reported for several medicinal plant species such as *Prunus serotina* Ehrh. (Espinosa *et al.* 2006). Ozaki (1993) also stated that callus of *Canavalia gladiata* (Jacq.) DC. cultured on MS medium fortified with NAA and BAP could produce the plantlets. Callus phase is important to distinguish the organogenesis or embryogenesis pathways (Narayanaswamy 1994).

Canavalia rosea is a halophyte species, which grows on salty coastal sand. This is an important characteristic for suitability of plant for soil stabilisation. Anatomical features are widely used in systematics approach, placing anomalous groups in appropriate position in the classification and indicating patterns of relationship that may be observed in morphological features (Essiett 2004). In *in vivo* and *in vitro* leaf of *Canavalia rosea,* different types and shapes of cell arrangement were observed in anatomical studies. In a broader aspect, the word histology is similar to microscopic anatomy, because its subject matter encompasses not only the microscopic structure of tissues but also cells and organ systems.

Every plant has a specific leaf venation, which is an important characteristic in the classification of various species. The *Canavalia rosea* leaf showed venation bipinate type with straight and branching veins. *In vivo* and *in vitro* leaves were amfistomic and stomata was observed on both adaxial and abaxial leaf surfaces. SEM observations revealed differences in stomata size and shape in *in vivo* and *in vitro* grown leaves. These observations correspond with result of a SEM study of leaves by Hazarika (2006). Trichomes were unicellular on both adaxial and abaxial leaf surfaces. Trichomes help to prevent the loss of water from leaf surfaces and offer a defense system against insects. Glandular and secretory hairs provide a chemical defense against latent herbivores as well. These results indicate that a systematic modification of morphology, anatomy and physiology was involved in the development of *in vivo* and *in vitro* grown plants.

CONCLUSION

The present work describes a simple and highly achievable regeneration protocol from direct shoot and root systems of different explants of *Canavalia rosea* with a reasonable survival rate in the *in vitro* growth culture. The adventitious origin of shoots from subepidermal cells of explants may aid in developing of a transformation method in this species. The observed anatomical and morphological characteristics were the key features that can be used to diagnose this plant.

ACKNOWLEDGMENT

The authors would like to thank the University of Malaya, Malaysia for the facilities and financial support provided (UMRG grant RP015B-14AFR and BKP grant BK024-2016).

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Recibido:22.08.2016
Aceptado:12.05.2017