IN VITRO MICROPROPAGATION AND MICROGRAFTING OF GUM ARABIC TREE [*Acacia senegal* (L.) Wild]

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ABSTRACT

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In order to find a reproducible method for *in vitro* multiplication of gum Arabic tree (*Acacia senegal* (L.) Wild). A protocol for *in vitro* micropropagatin and micrografting was developed at the laboratory of plant tissue culture, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan, during the period of October 2006 to October 2007 Multiple shoots were regenerated from cotyledonary node derived from 7-days-old *in vitro* raised seedlings and nodal segment derived from 12-months-old plant growing in a greenhouse. Explants were cultured on Murashige and Skoog (MS) medium supplemented with 0.5–5.0 $mgL^{-1}$of either benzyladenine (BA) or kinetin (Kn) alone or in combination with 0.5 mgL^{-1}α-naphthalene acetic acid (NAA). The maximum number of shoots per cotyledonary node (8.3 ±0.3) and nodal segment (5.3 ±0.7) explants were obtained on MS medium supplemented with 1.0 mg/l BA after 4 weeks of culture. *In vitro* regenerated shoots were either rooted *in vitro* on MS medium supplemented with auxins or micrografted on *in vitro* induced rootstock. Only 25% of the shoots formed roots after being transferred to MS medium containing 1.0 mgL^{-1}indole-3-butyric acid (IBA) after 28 days of culture under dark condition.The rate of successfully grafted shoots was influenced by both scion length and rootstock age. 100% successful graft was obtained with scion length of 3.0 cm and rootstock of 14-days-age. *In vitro* rooted shoots and successful grafts were transplanted to plastic pots containing autoclaved garden soil and sand (3:1), then hardened off and transferred to greenhouse where grown to maturity with 100% success. The success of the micrografting was independent of the nature and concentration of growth regulator used in shoot initiation medium and the time period for induction of shoots. This efficient plant regeneration system provides a solid basis for large scale reforestation and genetic improvement of this important multipurpose leguminous tree.

Key words: *Acacia senegal, in vitro* shoot regeneration, micrografting.

INTRODUCTION

Acacia senegal commonly known as "hashab" is a widespread leguminous tree, found in Sudano-Sahelian zone of Africa from Sudan to Senegal (Raddad, 2006). Its contribution towards environmental protection and economic development in the Sudano-Sahelian region is highly significant. It produces gum Arabic, which is used in a wide variety of products including candy, medicine, cosmetics and as an emulsifier in preventing precipitation of solids in soft drinks (Macrae and Merlin, 2002; Elfadl *et al.* 2000).

Sudan is known to have a higher density of *A. Senegal* with a uniform distribution of the tree in pure stands spread over fast geographic area places the country as the major producer of gum Arabic in the world (Beshai,1984). In Sudan gum Arabic grows in a broad band known as the ‘‘Gum belt’’ lies within the arid and semi-arid zone characterized by an erratic and scanty rainfall, in this zone the hashab tree is of vital importance to the permanent farming systems and gum Arabic production. However, due to environmental degradation and the fact that the regeneration rate of leguminous trees in natural habitats is low, the area of natural stands of this species has gradually declined (Seif El Din and Obeid, 1971). In response to this problem, large-scale planting programs with the help of local communities have been implemented since the early 1980s to restock the gum Arabic belt in order to curb desertification and to improve the gum Arabic yield and production in Sudan (Ballal *et al.* 2005).

Due to an effective vegetative propagation method is not yet developed, propagation through seed is currently followed for this program in Sudan. However, difficulty in obtaining selected seeds every year and storage of seeds as well as poor germination and death of young seedlings in natural habitats; do not always permit an adequate production of desirable selected seedlings for reforestation. In addition *Acacias* are out breeders, there is a high degree of genetic variations (Quoirin, 2003). Moreover, gum Arabic yield per tree varies from 0 to 1 kg per year (Sène, 1989), therefore, there is a great need for reproduction of individuals selected in nature for their abilities to produce large quantities of biomass and gum Arabic. Considering these aspects and the necessity of establishing a tree improvement program, propagation by tissue culture techniques is an alternative to supply genetically improved materials for reforestation.

A. senegal, *in vitro* micropropagation has already being reported (Badji *et al.* 1993). However, the regeneration protocols described in this report tend to be irreproducible and unsatisfactory. On the other hand, successful experiments were reported on *in vitro* micropropagation of number of *Acacia* species include *A. auriculiformis* (Mittal *et al.* 1989), *A.nilotica* (Dewan *et al.* 1992), *A.tortilis* (Detrez, 1994), *A.seyal* (Al-Wasel, 2000), *A.sinuata* (Vengadesan *et al.* 2002).

Many woody plants, economically important for timber and/or fruit production, are often difficult to root, both in conventional and *in vitro* propagation Damiano, C., and Monticelli, 1998). Recently, many attempts to overcome this problem have been carried out on fruit trees and woody species using micrografting. Application of micrografting technique for overcoming this problem has been successfully practiced for *Acacia tortilis* (Detrez, 1994). This article describes procedures for the *in vitro* clonal propagation, rooting and micrografting of *A. Senegal.*

MATERIALS AND METHODS

Plant material, explants preparation and culture methods

Seeds were collected from Takamol area, El Damazin, Blue Nile State, Sudan. The seeds were kept in an incubation room (25 ± 2°C, 40 % RH) until use. Seeds were pretreated with concentrated H_2SO_4 (95%) for 30 seconds then immediately washed by three changes with sterilized distilled water. After rinsing with distilled water, seeds were soaked in 70% (v/v) ethanol for 1 minute then immersed in a 100 ml solution of 20% (v/v) sodium hypochlorite for 10 minutes with continuous shaking then rinsed three times with sterilized distilled water. For germination the sterilized seeds were transferred to culture bottles containing half-strength MS (Murashige and Skoog, 1962) basal salt media.

Tow different types of explants were used in this study-

1- Cotyledonary node explant of 5.0 mm length excised from 7days-old *in vitro* raised seedlings.

2-Nodal segment explant of 5.0-8.0 mm length with one axiliary bud excised from 12-months-old plants growing in a greenhouse.

The nodal segment were surface sterilized by immersion for 15 min in 15 %(v/v) sodium hypochlorite solution plus two drops /100 ml of Tween–20. Explants were washed 3 times with sterile distilled water. Both explants were cultured in culture bottles containing MS basal media supplemented with benzyladenine (BA) (0.5- 5.0 mg L^{-1}) or Kinetin (Kn) (0.5- 5.0 mg L^{-1}) alone or in combination with NAA at 0.5 mg L^{-1}.

In vitro rooting and micrografting of shoots

For *in vitro* root initiation, the proliferating shoots (2-3 cm) were transferred to culture bottles containing half-strength MS hormone-free basal media or supplemented with three different concentrations (0.5, 1.0, 1.5 mg L^{-1}) of NAA, IAA or IBA under dark or light conditions.

For micrografting a cleft-graft (apex shoot-tip) was used. Rootstocks were prepared from 7 or 14 day-old *in vitro* raised seedlings as described for *A. senegal* seeds germination above. The shoots, axiliary buds and cotyledons were removed, and then root was decapitated with size of approximately 20 mm before grafting operation. After removing the shoot, the top cut surface was a longitudinally split to a depth of 50 mm until expose the stem vascular tissue.

Shoots of 28 days old and 1.5-3 cm length were sectioned from the multiplication medium and used as microscions. The basal end of the scion shoot was trimmed to a long "V" shape. Grafting was done by inserting the wedge of microscion gently into the vertical split on the rootstock so that at least part of the cambium layers of the 2 plants became aligned. The scion with a basal wedge was secured in the graft union by grafting between the two axes of the cotyledons and by compression of the hypocotyls halves.

Any axiliary shoots that emerging from cotyledonary node of rootstock were removed to avoid competition.

The effects of scion length (1.5-2 or 2.5-3 cm) and rootstock age (7 or 14days) were investigated. All the media used throughout this experiments were prepared by standard procedures, solidified with 0.7 % (w/v) Bacto agar and adjusted to pH 5.8 with NaOH before autoclaving at 121º C and 105 k Pa for 15 min. Plant growth regulators were added before autoclaving. All cultures were kept at 25º ± 2º C under warm-white fluorescent light at irradiance of 1000 lux in a 16-h (light) and 8-h (dark) photoperiod.

Statistical analysis

Results on the percentage of culture response, number of shoot per explants, rooting and grafting was observed at regular intervals. Data were collected from three independent experiments and subjected to analysis of variance. Means were compared with Duncan's Multiple Range Test (Duncan, 1955) and presented as average ± standard error (SE).

RESULT AND DISCUSSION

Multiple shoot induction

Direct multiple shoot induction is the useful means of production of plantlet from young or mature trees with a lower risk of genetic instability than by the other regeneration routes (Rao and Lee, 1986). In the present study efforts have been intensified to initiate *A. senegal* direct multiple shoot regeneration from cotyledonary node explants excised from 7days-old *in vitro* raised seedlings (Figure 1 A) and nodal segment explant excised from

twelve-months-old plant (Figure1 B). After a few days in induction medium containing growth regulators, both explants showed swelling at the cutting edges from which adventitious buds developed after 2 weeks in culture. However in induction medium without growth regulators (control) explants did not show any response. Evaluations were done after 28 days in culture. Explants 100% survival rate was obtained in all media tested. However, the frequency of *in vitro* multiple shoots formation and shoot length was significantly affected by types of explant and cytokinins as well as their concentrations (Table 1). Cotyledonary node explants cultured on induction medium containing growth regulators gave the highest number and longest *in vitro* regenerated shoot compared to those induced from nodal segment explant cultured on the same media. This result indicates that *A. senegal* cotyledonary node explants have a high regenerative capacity than nodal segment explant. Explant type has been shown to effect multiple shoot induction in a number of trees including *Dalbergia sisso* (Pradhan *et al.* 1998), *Pterocarpus marsupium* (Anis, 2005) *Albizia lebbeck* (Mamun *et al.* 2004), and *Albizia odoratissima* (Rajeswari and Paliwal, 2006).

Both cotyledonary node and nodal segment explants produced shoots when cultured on MS medium supplied with BA or kn alone or in combination with NAA (Figure1C and D). However, the mean number of shoots per explant and shoot length varied significantly with the varying type and concentrations of cytokinens in the induction medium. Our result showed that BA was able to induce the maximum number of shoots in both explants compared to kin (Table 1). Among the various concentrations of BA tested, 1.0 mg L^{-1}BA resulted in formation of the highest number of shoots from both cotyledonary node (8.3±0.3) and nodal segment (5.3±0.7) after four weeks of culture (Table 1). However, increasing the concentrations of both BA and kn beyond 1.0 mg L^{-1}evoked shoot proliferation, but the percentage response not declined. The superiority of BA has also been reported for other *Acacia* species (Mittal *et al.* 1989; Galiana *et al.* 1991; Dewan *et al.* 1992; Badji *et al.* 1993; Singh *et al.* 1993; Sahoo and Chand, 1998).

Inclusion of NAA (0.5 mg L^{-1}) in combination with kn or BA in the culture medium was not effective in enhancing shoot proliferation, but supported prolific callus growth at the basal end of both explants. The study revealed that exogenous auxin (NAA) was not essential to initiate shoot bud formation. However, the addition of cytokinin promoted the development of more shoots, thereby demonstrating the requirement of exogenous cytokinins for the sprouting of axillary buds. In consistent with this result, Vengadesan *et al.* (2002) have reported that when cotyledonary nodes explants of *Acacia sinuata* were cultured on MS medium containing a combination of BAP and auxins (NAA, IBA and IAA), the number of shoots was reduced but in turn produced basal callus.

In vitro rooting of shoots

For rooting, the proliferated shoots (2–3 cm height) developed from both explants were singled out from the parent tissue and cultured on half-strength MS medium supplemented with various concentrations (0.5, 1.0 or 1.5 mg L^{-1}) of IAA, NAA or IBA under light condition. After 28 days of culture on the rooting medium, there was no response of shoot cultured in half-strength MS medium with or without growth regulator under warm-white fluorescent light at irradiance of 1000 lux in a 16-h (light) and 8-h (dark) photoperiod. Similar results were also observed when *Pterocarpus marsupium* shoots inoculated in MS (1/2 and ¼ strength) supplemented with IAA, IBA and NAA (Anis, 2005). Because of the poor results of the first series of experiments, darkness treatment was tested; the same experiment was repeated under dark condition. The result showed that only medium containing IBA at the concentration of 1.0 mg L^{-1}induced 25% rooting. In this rooting medium, cultured shoots produced 1 root per shoot of 6.0 mm maximal length (Table 2) (Figure1E). The positive effect of dark treatment on rooting may be correlated to the faster metabolism of endogenous or exogenous auxins in the dark compared to the light (Maynard and Bassuk, 1987).

Most of the rooted shoots were originally regenerated on media containing kn and only a few shoots that regenerated on medium containing BA developed roots. Similarly, a number of authors (Kar *et al.* 1996; Polowick *et al.* 2004; Sarker *et al.* 2005) reported the poor rooting response for shoots that regenerated in BAP containing media.

Micrografting

Shoots that failed to develop roots were subjected to micrografting on rootstocks of *in vitro* germinated seedlings (Figure 2A-D). The rate of successfully grafted shoots was influenced by both scion size and rootstock age. Scion of 2.5-3 cm length gave the best results with 93% successful grafts, whereas only 73% successful grafts were obtained for the scion of 1.5-2 cm length (Table 3). For the rootstock age, the 14-days-age was found to be more efficient than 7-days-age (Table 4). That, all (100%) of rootstock aged 14 days grafted successfully while only 83% successful grafts were obtained for the rootstock of 7 days old. Similar observations were reported for sandalwood (Sanjaya *et al.* 2006). Removal of the cotyledons from the rootstock helped in insertion of scion and did not affect graft survival.

Although grafting was proved to be simple and easy to use, but relatively higher percentage of failed grafts (27%) was occurred with the young rootstock (7 days-old) and small scion (1.5-2.0 cm). This result is inconsistent with that obtained by Nelson (2006), who reported that the failure in grafting *Acacia koa* was due to the narrow stem diameter of rootstock and young stem tissue of scion which easy damaged. The result showed that, shoot necrosis was observed with both scion length as 7% at (2.5-3 cm) and 27% at (1.5-2 cm), while in rootstock aged 7 days was (14%). According to Sha *et al.* (1985) apical necrosis in shoots may be related to a mineral nutrients deficiency, e.g., Ca, resulting from the high levels of humidity in the container microenvironment.

This experiment demonstrated that micrografting is fast, effective and reliable and could be applied as an alternative to obtain healthy root system for the development of *A. Senegal* plants *in vitro* (Figure 1F). Successful cleft-grafting have been reported for *Acacia mangium* (Monteuuis, 1996) *A.tortilis* (Detrez, 1994), *Faidherbia albida* (Danthu, 2002).

Micrografting of *A. seneal* shoots overcomes many rooting problems and is independent of growth regulator treatment applied to the regenerated shoots. Moreover, micrografting of shoots results in both significant reduction in the regeneration period and a significant increase in the production of *A. seneal* regenerants. The propagated plantlets grew well and did not show morphological abnormalities (Figure 2E). The *in vitro* shoot regeneration procedure reported in this study is rapid, efficient and reproducible thus can be used for large scale reforestation and is expected to contribute to the future genetic manipulation studies of this important multipurpose tree legume.

Table 1. Effects of types of explant and different levels of cytokinens (BA, Kn) alone or in combination with 1-naphtalenacetic acid (NAA) on shoot multiplication of *Acacia. senegal* after 28 days of culture.

Plant growth regulator (mg L^{-1})			Survival (%)	Mean number of shoots (Mean ± SE)		Mean height of shoots (Mean ± SE)	
BA	Kin	NAA		Nodal Segment	Cotyledonary node	Nodal Segment	Cotyledonary node
0	0	0	100	1.5±0.6cd	1.3±0.3def	1.0±0.6efg	2.7±0.7a
0.5	0	0	100	2.0±0.0c	2.0±0.0d	0.8±0.0fgh	2.6±0.2ab
1.0	0	0	100	5.3±0.7a	8.3±0.3a	1.2±0.7def	2.7±0.6a
1.5	0	0	100	2.7±0.3b	5.3±0.7a	1.3±0.3cde	2.6±0.6ab
3.0	0	0	100	3.0±0.6b	0.7±0.3efgh	1.1±0.6def	0.9±0.4def
5.0	0	0	100	2.0±0.6c	1.0±0.6defg	1.3±0.6cde	0.8±0.3ef
0	0.5	0	100	1.5±0.6cd	3.3±0.7c	1.7±0.6ab	1.7±0.7bcd
0	1.0	0	100	2.0±0.0c	1.0±0.6defg	1.8±0.0a	1.2±0.7cde
0	1.5	0	100	1.7±0.3cd	1.3±0.9def	1.9±0.3a	1.7±0.7bcd
0	3.0	0	100	1.7±0.3cd	0.3±0.3fgh	1.8±0.3a	2.0±0.8bc
0	5.0	0	100	1.7±0.3cd	0.3±0.3fgh	1.6±0.3abc	1.7±0.7bcd
0	0	0.5	100	1.0±0.3ef	0.7±0.3efgh	0.7±0.3ghi	0.5±0.0f
0.5	0	0.5	100	1.3±0.3de	1.0±0.6defg	0.8±0.3fgh	1.5±0.5bcd
1.0	0	0.5	100	1.0±0.3ef	0.3±0.3fgh	0.6±0.3hi	0.5±0.0f
2.0	0	0.5	100	1.3±0.3de	0.3±0.3fgh	1.0±0.3efg	0.7±0.2ef
4.0	0	0.5	100	1.7±0.3cd	0.3±0.3fgh	1.4±0.3bcd	0.5±0.0f
0	0.5	0.5	100	1.7±0.3cd	1.3±0.3def	1.4±0.3bcd	1.2±0.7cde
0	1.0	0.5	100	1.0±0.0ef	1.7±0.3de	1.4±0.0bcd	2.3±0.9abc
0	2.0	0.5	100	1.0±0.3ef	0.7±0.3efgh	0.6±0.3hi	0.9±0.4def
0	4.0	0.5	100	2.0±0.7c	0.7±0.3efgh	1.3±0.7cde	0.9±0.4def

Means with same letter (s) in the same column are not significantly different at 5% using Duncan's multiple range test

Table 2. Effect of auxins on *in vitro* rooting of raised shoots *A. senegal* shoots after 28 days of culture in half-strength MS basal medium and under dark conditioned

Auxin (mg/L)		Number of shoot cultured	Survival (%)	Rooting (%)	Number of root per shoot	Root length (cm)
Control	0	20	100	0	0	0
IBA	0.5	20	100	0	0	0
	1.0	20	100	25	1	0.6
	1.5	20	100	0	0	0
IAA	0.5	20	100	0	0	0
	1.0	20	100	0	0	0
	1.5	20	100	0	0	0
NAA	0.5	20	100	0	0	0
	1.0	20	100	0	0	0
	1.5	20	100			

Table 3. Effect of scion length on successful grafts

Shoot length (cm)	Number of grafting	Necrosis	Failure	Successful grafts (%)	Successful (%)
1.5-2.0	15	2	2	73	100 (11/11)
2.5-3.0	15	1	0	93	100 (14/14)

Table 4. Effect of rootstock age on successful grafts

Rootstock age (Days)	Number of grafting	Necrosis	Failure	Successful grafts (%)	Successful (%)
7	15	2	0	86 (13/15)	100 (13/13)
14	15	0	0	100 (15/15)	100 (15/15)

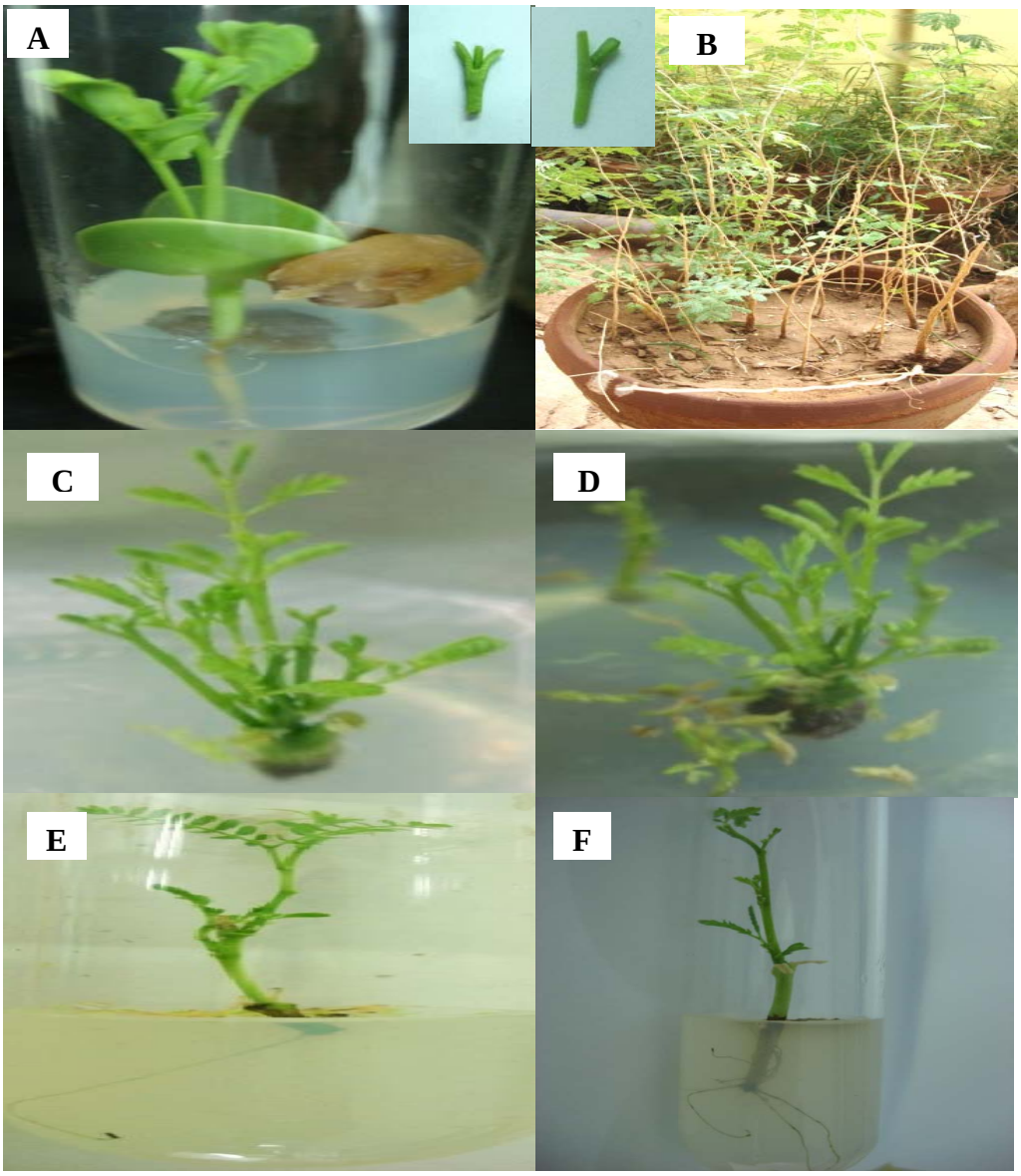

Figure 1. *In vitro* morphogenic responses of cotyledonary node and nodal segment explants of hashab tree [*Acacia Senegal* (L.) Wild]. (A) 7-days old in vitro raised seedling and 5.0 mm cotyledonary node explant. (B) 6 months-old seedling grown in greenhouse, and 5.0 mm nodal segement explant. (C) Multiple shoots differentiated on cotyledonary node cultured on MS medium supplemented with l.0 mgL^{-1} BA. (D) Multiple shoots differentiated on nodal segement cultured on MS medium supplemented with l. mgL^{-1} BA. (E) *In vitro* regenerated shoot rooted on MS medium supplemented with 1.0 mgL^{-1} IBA under dark conditioned. (F) *In vitro* regenerated shoot micrografted on scion of 3cm size and 14 days-old.

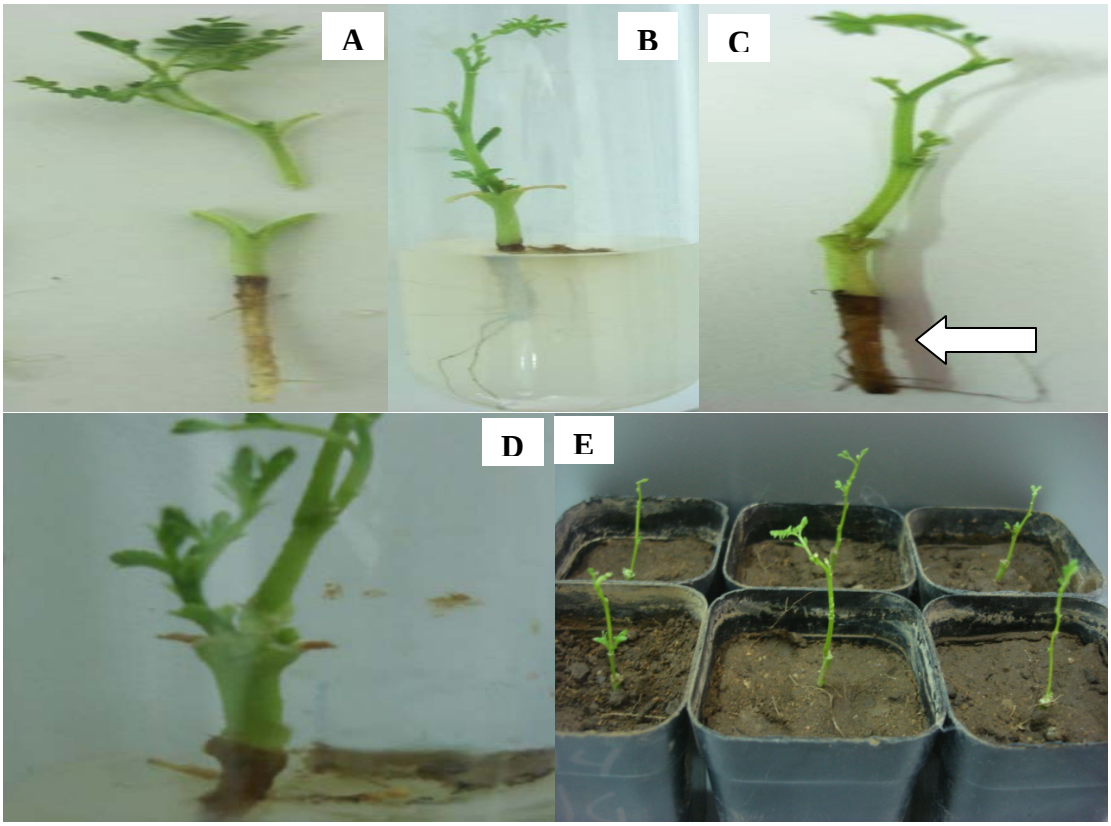

Figure 2. Stages of successful micrografting of *Acacia senegal*. (A) Scion with a 'V' shape end and rootstock with vertical split. (B) *In vitro* shoot after grafting. (C) Micrografted plant (arrow: the point of graft union) (D) Micrografted plant with new emerged axillary shoot, 2 weeks of culture. (E) Acclimatized plantlet in pots after 4 weeks of culture.

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