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Tissue Culture in Sudan Forest *In Vitro* Germination and Micro-Propagation of *Acacia tortilis* Subspp. *Spirocarpa*, A Multipurpose Forest Tree

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ABSTRACT

The paper highlights the importance of plant tissue methods and usage in producing planting materials of tree species, varieties and cultivars. Some local experiments of forest trees will be demonstrated such as; *in vitro* germination and micro-propagation *of Acacia tortilis* subspp *spirocarpa*, that shows a potential in shoot regeneration and callus formation after subcultures. The general aim of the paper is to highlighting benefit of tissue and organ cultures and researches achieved. Plantations regarding subspecies *A. tortilis* are scarce and their natural stands are declining at a rapid rate. Also, they exhibit a slow rate of growth and development although they are known as multi-purpose trees. The success in mass clone propagation through tissue culture from selected individuals might improve rate of growth and quality of selected traits and resulting in short-term gains. The seeds have been surface sterilized and then *in-vitro* germinated on Murashige and Skoog medium to produce healthy seedlings free of contaminants for micropropagation. Apical and epicotyls shoots were excised from 3-5-7week-old seedlings, cultured on modified MS basal medium supplemented with 0.10, 0.20 and 0.25 mg/l 1-naphthalene acetic acid (NAA) and 6- benzyl-aminopurine (BAP), separately and in combination. Callus was developed in cultures and new shoot plantlets rooted with 100 mg/l IBA giving 60 percent rooting and 40 percent rooting with 500 mg/l IBA. It is possible to develop a simple and feasible *in vitro* propagation protocol for production of *A. tortilis* subs. *Spirocarpa*; a multipurpose tree of economic and environment importance in Sudan

Key Words: Biotechnology, tissue culture, micropropagation, forest trees, acacias, *acacia tortilis* subspp.

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INTRODUCTION

Vegetative propagation by using tissue culture is a biotechnology process whereby large tree with desired traits could be launched in laboratory. Tissue culture is assumed to fulfill two goals of: Mass clone production where plantlets produced from tissues of embryos, cotyledons and other tissues from juvenile and mature plants (organogenesis or micro- propagation). Also use of callus or individual cell for cell suspension and protoplasts cultures. This will lead to tree improvement, hence, maximum genetic gain can be captured; manifested and new variations can be attained through biotechnology. In addition reforestation requires tree improvement and the stress has been towards production of multipurpose trees having genetically superior traits. The selected trees can be fast grow of improved dimensions increment, high quality of wood, disease resistance and environment tolerant *(Durzan, 1988)*. Plant tissue culture includes all forms of plant cultures grown aseptically from undifferentiated unit of protoplasts, callus to tissues and organized organs. Generally propagation of plant by seed is not justifiable for production of true- to-type species or cultivars of disease resistance, with high quality and yield; in Sudan we find decline and unstable production of gum Arabic *(Ali, 1997)*. However, it was been reported that individual variations amongst trees in seeds do not pose important problems for large plantations. But it could adversely affect standard of living of small farmers due to decreased yield found by a lower level of resistance to disease.

The use of tissue culture will enable production of forest trees with possible uniformity in growth, size and quality by avoiding or controlling the problem of variability among and within clones. More are; preservation of genotypes by use of clone bank, multiplication of desired genotypes in seed, research or breeding orchards; evaluation of genotypes and environment interaction through clone testing in operational programmes *(Vasil, 1990)*. Adding to; better understand of applications in medicine and public health, vector control, biochemical, cytological, physiological and pharmaceutical intricate processes *(Fowler, 1984 and Maurice, 1987)*; isolation and mass production of plants' molecules of active ingredient to be administered in defined doses *(Sasson, 1988)*. Therefore many plants possess active secondary or by product compound that forming initial step for drug production (United State Office of Technology Assessment, *OTA, 1983)*.

The main problem encountered in tissue culture of trees in field is to obtain explants free of contaminant due to difficulties manifested in surface sterilization of explants. Also metabolic processes and genetic factors affected various shoot stages of growth, development and callus formation (*Nehra and Kartha, 1994 and Ali, 1997*). However, for techniques of tissue culture to be commercially viable for forest trees, post-culture performance in field should be improved (*Jones and Van Staden, 1997*). Also if cost is considered economically feasible; it should encourage

introduction of tissue culture for production of improved seeds, seedlings of high yield and free-diseased plants as well as for other biotechnology processes.

Recently, intensive *in vitro* work and success has been achieved by production of plantlets from cultures from different explants of some trees. These achievements encourage application of biotechnology in trees such as; production of genetically improved plantlets for plantation programmes and of disease-free clones *(Cheliak and Roger, 1990; Ali, 1997 and Sascha and Robert, 2001)* and somatic embryogenesis work on *Acacia senegal* (L) Willd. *(Shahana and Cupta, 2000)*. However by embracing new concepts of biotechnology there is the possibility of time shortening or speed in genetic gain, improvement, earlier flowering and regeneration of forest plants *(Han et al., 1997)*. In addition application of biotechnology will add more advances in; genetic engineering of desired traits, a biotic stress tolerance and nitrogen fix genes. Also gene transformation, biodiversity, gene conservation and processing of forest non-wood secondary products such as modification of lignin composition *(Tzifra, 1998 and Ravikumar, 2001)*. In forest trees use of adventitious organ culture is a faster multiplication type that shows genetically apparent plants and axillary's bud multiplication is slower but, almost genetically stable as meristems shoot tip cultures.

The first work reported on tissue and organ cultures in forest trees was on shoot tips of *Acacia koa* by *Skoleman and Mapes (1976) and Duhoux and Davies (1985)* used cotyledon buds of *Acacia albids* (Faidherbia albida), *in vitro* micro-propagation of *A. senegal* seedlings by *Badji, et al. (1993)* and shoot regeneration from mature tree explants of *A. tortilis* by *Detrez (1994)*. In Sudan the work includes in-vitro micropropagation and callus formation of *Eucalyptus microtheca* by *Ali (1991)*, *in vitro* micro-propagation of *A. senegal* by *Salih (1994); Ibrahim (2000) and El-Tigani and Ali (2001)*, shoot regeneration from seedling explants of *A. tortilis* by *Ali (2007)*, shoot formation and rooting of shoots of *Acacia seyal* var *seyal* by *Altayab (2004)*, *in-vitro* propagation of *Grewia tenax* seedlings by *Ali, and Abd Alla (2003)* and *in vitro* propagation of *Silvadora persica* using single node by *Mohamed (2003)*. The work in tissue culture presented in this paper and conducted in Sudan institutes, started early since 1983 on some socioeconomic forest trees and shrubs but work on *Acacia tortilis* will be presented as an example. The main objective is to explore the possibility of developing a simple and feasible *in vitro* technique for high multiplication rates (MR) of uniform genotypes of *A. tortilis* subs. *spirocarpa*; a multipurpose tree of economic and environment importance in Sudan

MATERIALS AND METHODS

***In vitro* germination:**

Seeds of *A. tortilis* were treated with 50% (W/V) sulphuric acid for 45 minutes and washed in several changes of sterilized water. The seeds were dipped in absolute ethanol for 3-5 seconds; washed thoroughly by distill water; immersed in 20% (v/v) sodium hypochlorite solution containing 1.5% available chlorine for 20 minutes; then washed in four changes sterilized distill water. Murashige and Skoog's medium (MS) *(Murashige and Skoog, 1962)* was used as basal culture medium for shoot induction, elongation and root induction. The culture medium was supplemented with 2% sucrose, solidified by 0.8% agar and of pH 5.8.

Fifty sterilize seeds were aseptically placed on 10ml culture medium contained in culture tubes (140 x 2.50 mm). The set of experiment was incubated in culture room at 25 ± 2°C with specified light source. For *in-vitro* micropropagation, explants were taken from 21-day-old *in vitro* grown seedlings of *A. tortilis* (Figure 1). Apical and lateral shoots of 10-20 mm length were cultured vertically on 10 ml MS medium.1-naphthalene acetic acid (NAA) and 6-benzyl amino purine (BAP) were added before autoclaving. The concentrations used were 0.0, 0.10, 0.25, 0.20 mg/l BAP, NAA mg/l and combinations of BAP and NAA and the control is of MS medium without hormones. Explants were grouped in five replicates for each concentration then arranged in a randomized block design and incubated in culture room. Number of shoots from apical or axillary's bud was recorded for root initiation. For root induction regenerated shoots were excised when 10-20 mm long and placed vertically on the MS medium supplemented with 10 g/l, sucrose, indole-3-butyric acid (IBA) at the concentrations of 0.00, 0.05, 0.10 and 1.00 mg/l and 6 g/l agar with a pH 5.8. The number of rooted shoots and the developed callus were observed, recorded and evaluated.

RESULTS

The apical shoot explants developed in various hormone concentrations while showing significant decline with increase culture age. No significant difference was obtained in shoot development between apical and epicotyls explants in presence of growth regulators (BAP, NAA and BAP+NAA). Significant difference was obtained between 0.25 mg /l BAP (high number of shoots) and 0.20 mg/l NAA (low number of shoots) compared with the control (Table 2). In epicotyls cultures some explants survived and developed secondary shoots while in others the base in contact with the medium became swollen and produced callus. However, callus was formed at the cut ends of both apical and epicotyls cultures with varying intensity degrees. The highest intensity degree of callus was shown with NAA at 0.20 mg / 1 (Table 1) hence high concentrations of NAA significantly increased callus development in both cultures. In cultures without growth regulators (control) no callus was formed and shoot proliferation was significantly lower in epicotyls cultures compared to apical shoot ones (Figures 1A and B). However, shoots developed on cultures with callus of lower degrees of intensity. The subculture of proliferated explants on medium without growth regulators enhanced shoot growth and inhibited further development of callus (Figure 2).

Root formation was obtained on MS media containing 0.10 and 0.50 mg/1. On MS medium Rooting was occurred on MS basal media supplemented with various concentrations of IBA. Root formation was best at 0.1 mg/1 IBA (Table. 2) compared to higher concentrations that resulted in low rooting percent and poor performance.

Table 1: Effects of growth regulators and age on shoot regeneration and callus development of *Acacia tortilis* apical explants.

Treatment*(mg/l)	Age of explants (weeks)			
	3	5	3	5
	Average No. Shoot/ explants		Callus intensity	
0.25 BAP + 0.20 NAA	2.00 a	1.60 a	++	++
0.25 BAP	3.00 a	0.80 b	+++	-
0.20 NAA	1.80 b a	0.40 c	++++	++
0.10 NAA	1.50 b	0.40 c	++++	++
0.10 BAP	0.63 b	1.00 ba	++	+
0.10 BAP+0.10NAA	0.46 c	0.63 b	-	-
0.00	0.12 c	0.86 b	-	-
Effects of growth regulators and age on epicotyl's shoots regeneration and callus development of *Acacia tortilis* explants.				
0.25 BAP + 0.20 NAA	1.00 a	1.00 a	++++	++
0.10 BAP	0.60 b	0,95 a	++	+
0.25 BAP	0.46 b c	0.80 a	+++	-
0.10 NAA	0.50 b	0.20 c	++++	++
0.20 NAA	0.67 b a	0.78 ba	++++	++
0.10 BAP+0.10NAA	0.26 c	0.43 c	-	-
0.00	0.24 c	0.12 c	-	-

Means with different letter (s) are significantly different at 5% level using Duncan Multiple Range Test. Intensity of callus: (-) Nil; (++) moderate; (+++) high; (++++) intense.

Table 2: Root percent on MS medium by use of Indole-3-butyric acid (IBA / mg/1) of regenerated shoots of *Acacia tortilis*.

Rooting %	Number of excised plantlets	IBA (mg/l)
0	4	0.00
0	4	0.05
60	5	0.10
40	5	0.50
0	6	1.00

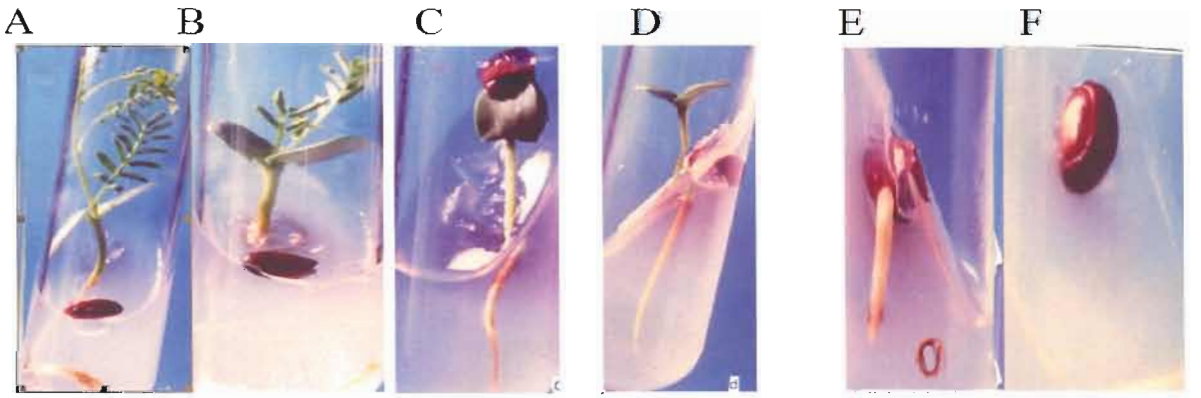

Figure 1A: Germination of *A . tortilis* subs, spirocarpa , stages of *in-vitro*.

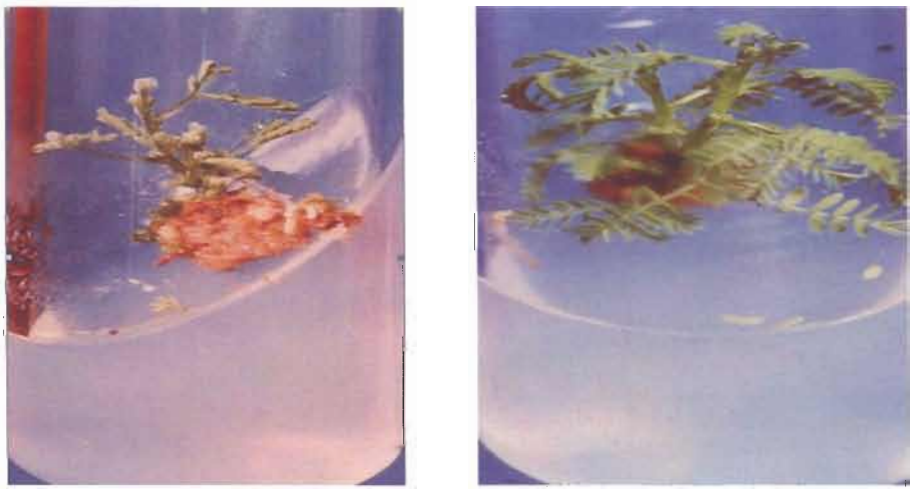

Figure 1B: Callus and shoot proliferation of *A. tortilis* of 3 plantlets.

Figure 2: The subculture of proliferated explants on medium without growth regulators enhanced shoot growth and inhibited further development of callus.

DISCUSSION

In this work high potential for shoot multiplication and callus formation was demonstrated providing promising approach for *in vitro* mass propagation of *Acacia tortilis*. The intensity degrees of formed callus on cultures of apical and epicotyls explants were affected by addition of NAA to culture medium. *Mittal, et al. (1989)* obtained similar results with plantlets of *Acacia auriculformis*. It seems that use of high concentration of NAA (0.25 mg/1) causes prolific callus growth that results in failure of explants to produce plantlets. This could be attributed to the ways of shifting the metabolic processes of the cultured explants to be redirected to inhibit organogenesis *(Bonga, 1981)*. However, low concentration of NAA (0.1 mg/1) was used by *Macrae (1994)* for shoot and bud formation of *Acacia tortilis*. On the other hand, BAP and the combinations of BAP and the NAA significantly affected shoot regeneration with low callus intensities. Similar result was obtained by *Macrae (1994)* work on cotyledons and nodes of *Acacia tortilis*. Hence, it may assumed that, BAP alone was effective in inducing differentiation of shoot buds, proliferation and it probably inhibits the effect of NAA on callus formation.

Continuous presence of growth regulators in the culture medium resulted in inhibition of bud growth and shoot proliferation of both types of explants. Similar observations reported by *Abdullah, et al. (1989)* on Calabrian pine plantlets propagated *in vitro* and they used pulse treatment to overcome this phenomenon. This has been attributed to competition exerted by growing tissues in direct contact with nutrient medium and metabolic processes. However, subcultures of regenerated shoots on media without growth regulators positively affect shoot elongation and inhibited callus formation. The variations showed by apical and epicotyls explants in shoot formation and bud induction were similar to results obtained by *Carolina, et al. (1990)* with regeneration of plantlets of *Pinus canariensis*. This phenomenon had more or less limited by the type, morphology and the physiological age of explants or the differences in genetic capacity among mother plants and of organs between various parts of the plant. Hence, it seems that, the source of explants probably affected the natural capacity of explants to regeneration.

Rooting was occurred on MS basal media supplemented with various concentrations of growth regulators. Root formation was best at 0.1 mg/1 IBA (Figure 2) compared to higher concentrations that resulted in low rooting percent and poor performance. This finding was similar to that obtained by *Jones, et al. (1990)* on a number of Acacia species. It seems that the incidence of rooting could be due to influences by auxins interaction with chemicals, growth cofactors or auxins synergists such as phenol compounds.

High capacity for shoot multiplication and callus formation was demonstrated providing promising approach for *in vitro* mass propagation of *A. tortilis*. The intensity degree of formed callus on cultures of apical or epicotyls explants were affected by addition of NAA to culture medium similar to work on *A. auriculformis* by *Mittal, et al. (1989)*. The use of juvenile explants and callus formation was inline with most work conducted on woody plants specifically acacias. On the other hand it seems that use of high concentration of NAA (0.2 mg/1) causes prolific callus growth that results in suppress or failure of explants to produce plantlets. This could be attributing to ways of shifting metabolic processes of cultured plants to be redirected to inhibit organogenesis *(Abdullah et al., 1989)*. On other hand, BAP and (BAP + NAA) combination significantly affect shoot regeneration with low degree of callus intensity in multiplication rate (MR) of ×2 and ×3. It is similar to result by *Macrae (1994)* work on cotyledons and nodes of *A, tortilis* and other work on acacias. Hence, it may be assumed that, BAP alone was effective in inducing differentiation of shoot buds, proliferation and it probably inhibits the effect of NAA on callus formation.

CONCLUSION

It is possible to maintain *Acacia tortilis* callus for long periods as well as a number of regenerated shoots could be sub-cultured to give mass propagules from a single excised apical shoot. The whole shoot tips of *Acacia tortilis* can be used to produce a number of secondary shoots that provide a genetic stability of desired products Work should be continued to determine the maximum multiplication rate and to produce well developed root system. Moreover, continued work by the use of biotechnology will contribute in improving vegetative propagation of forest trees and playing an important role breeding programmes.

RECOMMENDATION

Therefore research on socioeconomic, endangered trees and shrubs should be encourage and continued. Fast growing tree species is a priority as other aspects related to energy. Moreover, continued research works on biotechnology aspect contribute to arrest deforestation, soil deterioration and improve application for fruitful utilization of forest

plants products. Therefore the prospective outputs and the perception that high returns are obtained should be added to support application of biotechnology in agriculture and specifically forestry sector. Tissue culture laboratory has been established in 2008 at Forestry Research Centre located at Soba which expected to add more to existing work in forest sectors.

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