African Journal of Biotechnology Vol. 11(43), pp. 10098-10107, 29 May, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.331
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Regeneration from embryogenic callus and suspension cultures of the wild medicinal plant *Cymbopogon schoenanthus*

Ahmed A. El-Bakry* and Asmaa M. Abdel-Salam

Department of Botany, Faculty of Science, Helwan University, Ain Helwan, Cairo 11795, Egypt.

Accepted 25 April, 2012

Somatic embryogenesis and plant regeneration from both callus and suspension cultures of the wild medicinal plant *Cymbopogon schoenanthus* subsp. *proximus* has been achieved. The species is rare and confined in its distribution to Africa. A range (0.5 to 8 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D) for the induction of embryogenic callus from seed cultures were used. Results show that 1.0 and 4.0 mg/l 2,4-D gave a 90 to 100% frequency of embryogenic callus containing mature embryos within three months of culture. Testing the effect of phosphorus and nitrogen concentrations on somatic embryogenesis from callus cultures showed that high phosphorus and low nitrogen concentrations enhanced embryo induction. Low NH_4NO_3 enhanced growth and maturation of somatic embryos, while low KNO_3 enhanced germination and shoot production. Suspension cultures were initiated from embryogenic callus on 0.5, 1.0 and 2.0 mg/l 2,4-D, then plated on 3 different combinations of 2,4-D and 6-benzyl adenine (BA). Mature embryos were highest on both 0.5 and 1.0 mg/l 2,4-D (X = 15.8, 17.3), while shoot germination was enhanced by using BA in the regeneration media. The effect of high phosphorus concentration in the culture media was significant on both embryo induction and early maturation (X = 41). Lower ammonium nitrate concentrations enhanced growth and maturation of embryos. Both embryo maturation and the germination of shoots after 6 and 8 months of plating were best on low ammonium nitrate (X = 36.8 and 27.6 shoots, respectively). The presented embryogenic system will be of value for the clonal propagation, *ex situ* conservation and production of bioactive compounds from this threatened plant species.

Key words: Somatic embryogenesis, gramineae, *in vitro* culture, proximol, micropropagation, plant conservation, nitrogen, phosphorus.

INTRODUCTION

The genus, *Cymbopogon* Spreng. (Gramineae) comprises about 40 species distributed in the old world tropics and subtropics, introduced to tropical America. A number of species are well known for their medicinal and economic importance worldwide (Boulos, 2005). *Cymbopogon nardus*, *C. martini*, *C. citratus and C. flexuosus* are the source of palmerosa, citronella, lemon-grass and oil of Malabar, respectively. *C*. *jwarancusa* is used as condiment and for medicinal purpose. *C*. *schoenanthus* is represented by subsp. *schoenanthus,* distributed in Egypt, Africa north of the Sahara and in Arabia, as well as, by subsp. *proximus* distributed in Egypt, Africa south of Sahara from Mauritania to Ethiopia and Kenya. Both subspecies are known for their folk medicine uses and medicinal importance. Phytochemical composition of *C*. *schoenanthus* subsp. *schoenanthus* showed the presence of piperitone, delta-2-carene, limonene, beta-phellandrene, delta-terpinene and α–Terpineole (Yentema et al., 2007; Khadri et al., 2008). *C*. *schoenanthus* subsp. *proximus* has been shown to contain principle cryptomeridiol (Locksley, 1982), proxy-madiol, 5α-hydroxy-β-eudesmol, 1β-hydroxy-β-eudesmol, 1β-hydroxy-α-eudesmol, 5α-hydroperoxy-β-eudesmol, 7α,11-dihydroxycadin-10-(14)-ene (EL-Askary et al., 2003). The plant is used extensively in folk medicine

*Corresponding author. E-mail: ael_bakry@yahoo.com. Tel: 01006607761. Fax: 20225552468

(Batanouny et al., 1999), against influenza, some neurotic diseases, for colic, fever, H-diabetes, as diuretic, stomachic, lower blood pressure, commetringent, carminative, sudorific and anti-rheumatic (Boulos, 1983). It is used as renal antispasmodic agent (Elgamal and Wolff, 1987), anti-hyperglycemic and used to alleviate liver and renal damage caused by allaxon-induced diabetes in rats (Sheweita et al., 2002). Due to its efficiency as a diuretic and a renal antispasmodic agent, and for the expulsion of ureteric calculi, the medicinal product PROXIMOL (Halphabarol) was produced (Ministry of Health, Egypt, product registration #24526) which depends completely on plant material collected from the wild. The hyper arid climatic conditions in the natural habitat of the species and the overcollection from the wild for both folk medicine and medicinal uses are affecting the growth and rejuvenation of the species in the wild. This is reducing the natural populations to a very limited geographical distribution and is leading to minimizing the genetic variation of the species that may shortly lead to its extinction. *In vitro* cultures of the species may provide a good approach towards the micropropagation, *ex situ* conservation and maintaining its genetic polymorphism. *In vitro* cultures for a number of *Cymbopogon* species have been studied. Regeneration through somatic embryogenesis has been carried out for *C. flexousus, C. pendulus*, *C. winterianus* (Nayak et al., 1996; Bhattacharya et al., 2009; Dey et al., 2010). Micropropagation, through seed culture has been studied in *C. martinii, C. winterianus* and *C. citratus* (Sreenath and Jagadishchandra, 1982, 1987; Tapia et al., 2007).

In vitro cultures for both subspecies of *C. schoenanthus* (subsp. *schoenanthus* and *proximus)* have not been reported so far. The present work reports regeneration from callus and suspension cultures of *C. shoenanthus* subsp. *proximus,* a medicinally important plant species that is endangered, due to hyper arid conditions and overcollection in the natural habitat. The study also shows the control of different stages of somatic embryogenesis through growth regulators (2,4-D and BA), phosphorus concentration and different nitrogen sources.

MATERIALS AND METHODS

Inflorescences of *C. schoenanthus* subsp. *proximus* were collected from Aswan (South Valley), Egypt in March 2006 and 2008 and stored in paper bags at 25°C. Seeds were separated from the inflorescence immediately before sterilization.

Regeneration from callus culture

Effect of 2,4-D on total and embryogenic callus production

i) Leaf explants culture from *in vitro* germinated seedlings

***In vitro* seed germination:** Seeds were collected in cheese cloth and rinsed under running tap water for 15 min, then washed with double distilled H_2O (ddH_2O). Seeds were then treated in 95% ethanol for 1 min, followed by immersion in 20% Clorox (NaOCl 5.25%) for 20 min. Under aseptic conditions, seeds were washed 3 times with sterile ddH_2O (3 to 5 min. each). Sterile seeds were cultured on half strength Murashige and Skoog (1962) media (4 seeds/magenta) supplied with 3% sucrose, pH adjusted to 5.8. Media was gelled using 2 g/l phytagel. Incubation was at 25°C, under 8 h of white fluorescent light.

Experiment 1: The basal 0.5 cm cross section of the outer leaves of 14 days old germinated seedlings were excised and cultured on MS media. Different 2,4-D concentrations (0.5, 1.0, 2.0, 4.0, 8.0 mg/l) and BA (0.0, 0.2, 0.5, 1.0 mg/l) were used in a factorial completely randomized design (CRD). For each treatment, 4 replication plates, 5 explants/plate were cultured.

Experiment 2: The outer 3 leaves from each seedling were dissected, then divided into 4 zones; a, b, c, and d from the bottom to the top. Explants 0.5 cm were cultured separately for both leaves and explants zone. MS media with 0.5 mg/l BA and different concentrations of 2,4-D (1.0, 2.0, and 4.0 mg/l) was used. For each treatment, 4 replication plates, 5 explants/plate were cultured.

ii) Seed culture

Sterile seeds were cultured on MS media supplemented with different 2,4-D concentrations (0.5, 1.0, 2.0, 4.0, 8.0 mg/l) and BA (0.0, 0.2, 0.5, 1.0 mg/l) in a factorial CRD. For each treatment, four plates were cultured (5 seeds/plate).

Effect of different phosphorus and nitrogen concentrations

Sterile seeds were cultured on MS media supplemented with 1 mg/l 2,4-D and 0.5 mg/l BA for 2.5 months, with one subculture after 1 month. Second subculture was carried out onto MS, containing different concentrations of potassium phosphate monobasic: 1.7, 3.4, 5.1, 8.5 g/l (corresponding to 1.0, 2.0, 3.0, 4.0 × of MS media concentration), potassium nitrate at 4.75, 9.5 g/l (corresponding to 0.25 × and 0.5 × of MS media) or 4.125, 8.25 g/l ammonium nitrate (corresponding to 0.25 × and 0.5 × of MS media). Media was supplemented with 0.25 mg/l 2,4-D and 0.125 mg/l BA. For each treatment, 4 replication plates, 5 explants/plate were carried out in a factorial CRD. Three subcultures were carried out after 4.5, 6 and 7.5 months. At 9 and 10.5 months, 5 ml MS media, containing 0.5 mg/l BA and 2% sucrose were added to each plate. Shoots were rooted on media containing 6% sucrose and 0.01 mg/l NAA. Percentage of callus formation, embryogenic callus, number of somatic embryos, number of maturing embryos, and number of shoots were recorded at 2, 4.5, 6, 7.5, 9, 10.5, and 11.5 months of culture. For all callus culture experiments, incubation was at 25°C under cool white fluorescent light, for a photoperiod of 8/16 h for the first 2.5 month of culture, followed by 16/8 h photoperiod till the end of the rooting stage. All cultures were examined and screened under OLYMPUS SZX-7 (Japan) zoom stereomicroscope.

Regeneration from suspension cultures

Effect of different 2,4-D concentrations

Sterile seeds were cultured on MS media supplemented with different concentrations of 2,4-D (0.5, 1.0, 2.0 mg/l) in addition to 0.5 mg/l BA (D2B2, D3B2, D4B2, respectively). Subculture onto media of the same composition after one month was carried out.

Suspensions were initiated by inoculating 0.2 g tissue into 20 ml media of the same composition and placed on a gyratory shaker at 110 rpm. Three flasks from each treatment were cultured. Five hundred (5 ml) of the same media composition were added after 10, 20, 30, 40 and 50 days from inoculation. After 60 days, 3 ml from the suspension culture were plated onto MS solid media containing: (1) Same concentration of growth regulators; (2) $^1/_4$ growth regulators concentration and; 3) BA 0.5 mg/l (SU3). Four replication plates from each treatment were carried out. Two subcultures, after 2.5 and 4 months of plating were carried out onto MS media supplemented with 0.01 mg/l BA and 3% sucrose. Five hundred (5 ml) of MS media supplemented with 0.01 mg/l 2,4-D and 0.1 mg/l BA were added after 5.5 and 6 months. Regenerated plantlets were transferred to hormone free media for rooting. Percentage of callus and embryogenic callus formation, number of embryos, maturing embryos and germinating shoots were recorded for 2.5, 4.0 and 6.0 months old cultures.

Effect of different phosphorus and nitrogen concentrations

Sterile seeds were cultured on MS media supplemented with 1 mg/l 2,4-D, 0.5 mg/l BA and subcultured after 1 month onto media of the same composition. Suspension cultures were initiated after 2.5 months by inoculating 0.5 g of callus tissue into 50 ml of MS media containing different concentrations of potassium phosphate monobasic: 1.7, 3.4, 5.1, 8.5 g/l, potassium nitrate 4.75, 9.5 g/l or ammonium nitrate 4.125, 8.25 g/l. All media contained 0.25 mg/l 2,4-D and 0.125 mg/l BA. Cultures were placed on a gyratory shaker at 110 rpm. For each treatment, 3 replication flasks were cultured. Three additions, 5 ml each, of media of the same composition were carried out after 20, 30 and 40 days from inoculation. After 50 days, 3 ml from suspension cultures were plated onto MS media with the same N and P concentrations, in presence of 1 mg/l 2,4-D and 0.5 mg/l BA. For each treatment, 4 replication plates were carried out. Three subcultures were carried out every 2 months onto the same N and P treatments. The last medium contained 1% each of sucrose, plus 1% maltose or 1% lactose. Healthy shoots were transferred to hormone free media for rooting. Explants were screened for percentage of callus formation, embryogenic callus, number of embryos, number of maturing embryos, and number of shoots after 2.0, 4.0, 6.0 and 8.0 months. OLYMPUS (Japan) stereomicroscope was used. All suspension cultures were incubated at 25°C in the dark. Plated suspension onto solid media were incubated at 25°C under cool white fluorescent light with a photoperiod of 8/16 h for 2 months, then transferred under 16/8 h photoperiod for the remaining culture period. Acclimatization was carried out in 15 cm plastic pots containing, 1:1 mixture of sand and peat moss. Incubation was at 25°C and 16 h photoperiod for 3 weeks.

Statistical analysis

Results were statistically analyzed by a factorial analysis of variance in completely randomized design according to the procedure outlined by Snedecor and Cochran (1981). Means were compared by multiple range test (Duncan, 1955).

RESULTS

Regeneration from embryogenic callus cultures

Effect of 2,4-D and BA

Somatic embryogenesis from leaf explants: Basal sections of leaf explants cultured on the experimental range of 2,4-D and BA, all failed to induce the growth of any callus over a period of 2 months. On culturing 4 different zones from the leaves of 2 weeks old *in vitro* germinated seedlings onto MS media with different concentrations of 2,4-D and BA, all explants failed to form callus except the explants from 1st outer leaf and 'c' zones. Culture media contained 2 mg/l 2,4-D and gave a 5% frequency of embryogenic callus formation.

Somatic embryogenesis from seed callus: Mature seeds cultured on media containing different 2,4-D concentrations in a factorial design with BA were screened after 2 and 3 months to determine the frequency of callus formation, embryogenic callus and mature germinating embryos.

ANOVA for callus formation was non-significant for the different growth regulators used and for the culture age. A 100% frequency of callus formation was obtained on a number of media as D1B0, D1B2, D3B0 and D4B2 (Figure 1a) after 2 months in culture. 3 months old culture showed a 100% callus formation in the absence of BA, as in D1B0 and D3B0. At higher 2,4-D concentrations, the presence of BA was necessary to obtain a 100% frequency as in D4B2 and D5B3.

ANOVA showed that treatments were significantly different at both 5 and 1%, after 2 and 3 months culture, for the frequency of embryogenic callus formation. The mean frequency of embryogenic callus formation was 67.4 and 83.2% after 2 and 3 months of culture. D2B2, D3B3 and D4B2 gave the highest embryogenic callus frequency of 90% and 80%, respectively after 2 months. After 3 months, a 100% frequency was on D4B2 and D5B2, followed by D4B1, D3B2, D3B1, D2B2 and D1B0.

Typical mature embryos of *Cymbopogon* starting to germinate appeared only during the 3rd month of the culture history. Highest frequency of 80% was on D4B2, followed by D5B3 (73%) and D2B2 (70%) (Figure 1e). The presence of BA, as well as, the ratio between 2,4-D and BA seems to control the growth and maturity of the embryos. Different developmental stages until whole plant regeneration are shown in Figure 2.

Effect of different phosphorus and nitrogen concentrations

For early somatic embryo numbers, high potassium phosphate monobasic (4×) concentration gave higher mean value 4.7 in the early stage, after 4.5 months. But 0.25× and 0.5× potassium nitrate were the most effective after 7.5 months, giving mean number of 0.66, 0.16 and 0.25×, ammonium nitrate gave higher mean values after 9 months of 0.83 (Table 1). ANOVA showed significant differences between treatments at both 5 and 1%, after 7.5 months between culture media.

Mature embryos growing on 0.5× ammonium nitrate containing medium, gave higher mean value after 4.5

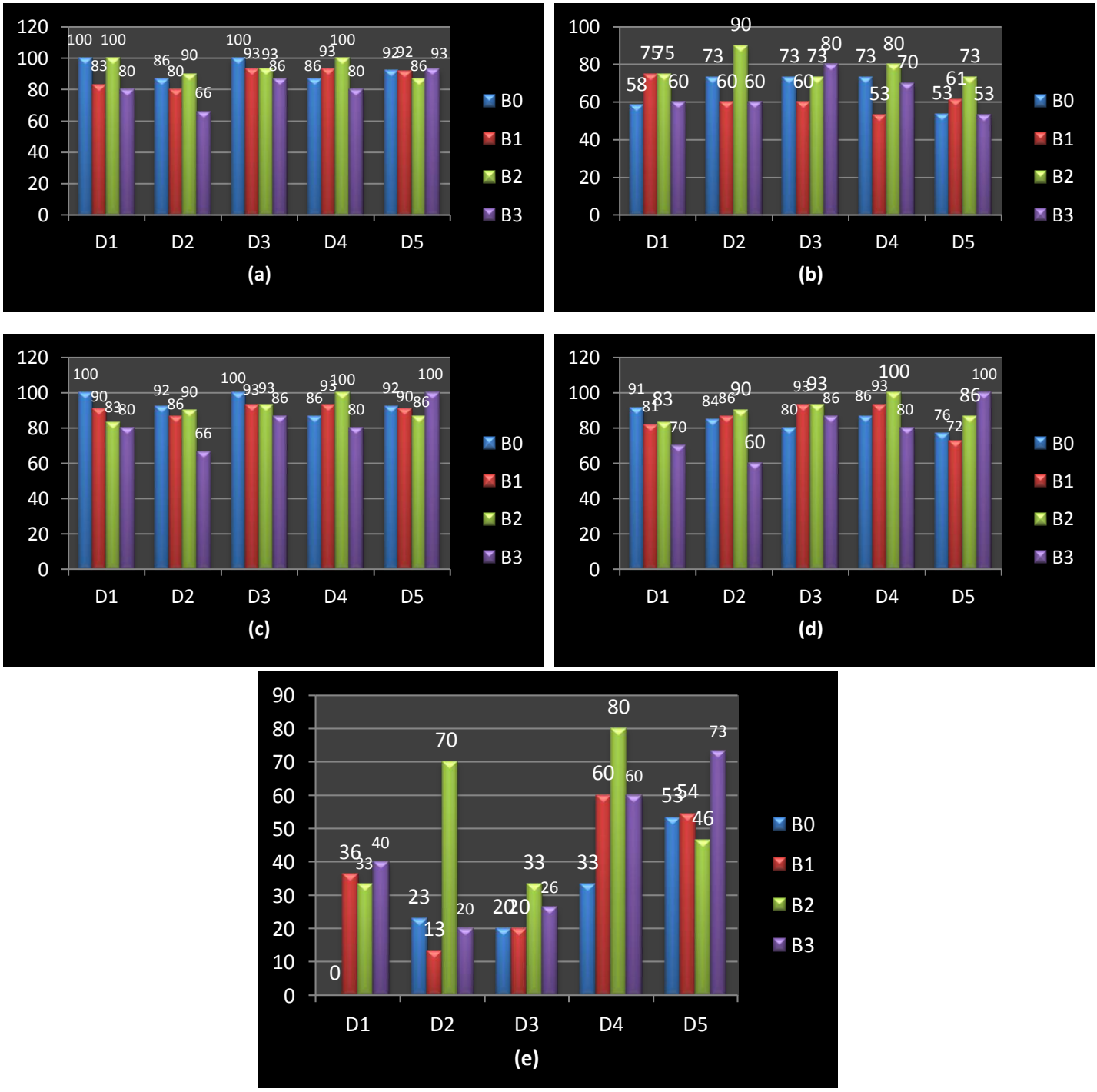

Figure 1. Effect of 2,4-D concentrations (D1 = 0.5; D2 = 1; D3 = 2; D4 = 4; D5 = 8 mg/l) and their interaction with BA (B0 = 0.0; B1 = 0.2; B2 = 0.5; B3 = 1 mg/l) on the frequency of (a) Callus formation; (b) Embryogenic callus after 2 months in culture; (c) Callus formation; (d) Embryogenic callus after 3 months in culture and; (e) Frequency of mature embryos after 3 months.

months (30.8), but control (1×) gave higher values after 6 and 7.5 months (27.16, 30.33, respectively) (Table 2). ANOVA showed significant difference between different culture media, at both 5% and 1%, after 10.5 months.

Shoot numbers for 0.25× and 0.5× potassium nitrate treatments gave higher mean values at 6 months (1.67, 1.67) and 10.5 months (18.0 and 8.5) (Table 3). ANOVA gave non-significant difference for different culture media and culture age. Results show that somatic embryos induction required low concentrations of potassium nitrate

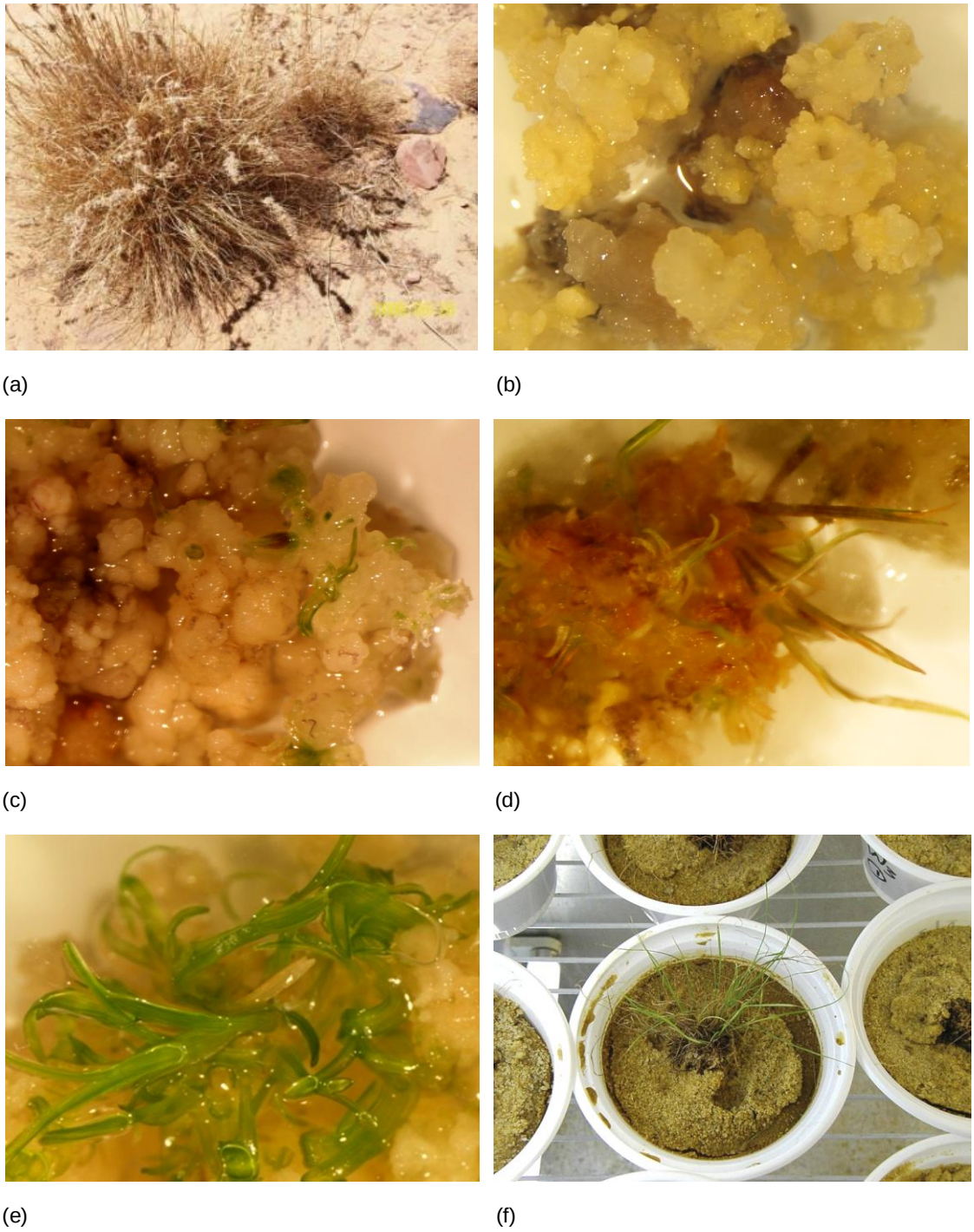

Figure 2. Regeneration from callus cultures: (a) Wild plants growing at Aswan, South of Egypt. Mature inflorescences were the seed source for the present study; (b) Early formation of embryogenic callus; (c,d,e) Different stages of maturation and germination of somatic embryos from embryogenic callus and; (f) Whole plants regenerated after acclimatization.

and ammonium nitrate, also a higher concentration of phosphorus was effective in earlier stage, but the effect was non-significant in later ones. For embryo maturation, a lower concentration of ammonium nitrate (0.5×) was found to be the most efficient with different culture ages. Healthy shoots with higher mean numbers were produced with lower (0.25× and 0.5×) potassium nitrate concentrations.

Table 1. Mean number of somatic embryos from callus cultures at different culture ages (4.5, 6, 7.5, 9 and 10.5 months). MS media was supplemented with different concentrations of potassium phosphate monobasic (P1 = 1.7; P2 = 3.4, P3 = 5.1; P4 = 8.5 g/l), different potassium nitrate concentrations (A1 = 4.75; A2 = 9.5 g/l) or different ammonium nitrate concentration (B1 = 4.125; B2 = 8.25 g/l).

Parameter	4.5	6	7.5	9	10.5
P1	0.55+0.55	0.00+0.00	0.00+0.00	0.00+0.00	0.00+0.00
P2	0.00+0.00	0.00+0.00	0.00+0.00	0.00+0.00	0.00+0.00
P3	0.11+0.11	0.44+0.44	0.00+0.00	0.00+0.00	0.00+0.00
P4	4.76+2.99	0.00+0.00	0.00+0.00	0.00+0.00	1.33+1.33
A1	0.33+0.33	0.16+0.16	0.66+0.33	0.32+0.16	0.00+0.00
A2	1.33+1.33	0.66+0.66	0.16+0.16	0.00+0.00	0.00+0.00
B1	3.83+3.83	0.00+0.00	0.00+0.00	0.83+0.83	0.00+0.00
B2	0.16+0.16	0.66+0.66	0.00+0.00	0.00+0.00	0.00+0.00

Table 2. Mean number of mature embryos from callus cultures at different culture ages (4.5, 6, 7.5, 9 and 10.5 months). MS media was supplemented with different concentrations of potassium phosphate monobasic (P1 = 1.7; P2 = 3.4; P3 = 5.1 and P4 = 8.5 g/l), different potassium nitrate concentrations (A1 = 4.75; A2 = 9.5 g/l) or different ammonium nitrate concentration (B1 = 4.125; B2 = 8.25 g/l).

Parameter	4.5	6	7.5	9	10.5
P1	15.88+5.45	27.16+12.77	30.33+18.93	9.00+7.32	0.00+0.00
P2	6.33+0.38	3.55+0.73	2.22+1.74	7.16+4.04	0.00+0.00
P3	13.99+4.48	9.55+4.23	8.99+3.94	0.99+0.19	0.00+0.00
P4	4.19+0.43	2.33+2.33	2.22+1.17	1.11+1.11	3.33+1.45
A1	15.5+8.73	8.33+7.08	2.32+1.16	9.63+2.42	6.43+1.69
A2	7.66+6.45	0.16+0.16	4.00+2.64	7.66+3.24	0.00+0.00
B1	8.66+3.37	14.00+2.56	7.00+1.89	2.16+1.30	1.00+1.00
B2	30.83+25.41	24.38+12.41	18.00+10.39	15.83+4.66	2.50+2.50

Regeneration from embryogenic cell suspension cultures

Effect of different 2,4-D concentrations

The effect of different 2,4-D concentrations on somatic embryos induction, maturation and germination was studied when suspensions were initiated from callus established on MS media with different concentrations of 2,4-D and maintained on media of the same composition. Tissues from each treatment were plated onto MS media with full concentration of growth regulators, $^1/_4$ growth regulator concentrations or 0.5 mg/l BA only (SU3). The experimental design is aimed at testing the effect of different 2,4-D concentrations and period of exposure in combination with BA.

Mean number of somatic embryos recorded were highest with D3B2 plated on $^1/_4$ growth regulators concentrations (3.66), after 2.5 months (Table 4), but D2B2 plated on full growth regulators concentrations gave higher values after 4 and 6 months (6.2 and 2.4). ANOVA gave significant difference at 5% in the 4th month and gave significant difference at both 5% and 1% at 6th month for plating media.

Mature embryos gave higher values with D2B2 plated on full growth regulators concentrations after 2.5 months (18.67). After 6 months, both D2B2 and D3B2 plated on full concentration of growth regulators gave higher values (15.8 and 17.3). ANOVA gave significant difference at both 5 and 1% after 6th month for plating media, and gave significant difference at 5% for combination between initial media and plating media.

For shoot number, media containing 0.5 mg/l BA recorded higher values after 2, 4 and 6 months (4.0, 2.6 and 5.0). ANOVA gave significant difference at 5% between different plating media and culture period.

Effect of different concentrations of phosphorus and nitrogen

The numbers of somatic embryos, mature embryos and shoots showed a diversity of mean values for the different media treatments used and for different culture ages. For somatic embryos, a higher concentration of potassium phosphate monobasic (4×) gave high mean value of (6.0)

Table 3. Mean number of shoot numbers from callus cultures at different culture ages (4.5, 6, 7.5, 9 and 10.5 months). MS media was supplemented with different concentrations of potassium phosphate monobasic (P1 = 1.7; P2 = 3.4, P3 = 5.1; P4 = 8.5 g/l), different potassium nitrate concentrations (A1 = 4.75; A2 = 9.5 g/l) or different ammonium nitrate concentration (B1 = 4.125; B2 = 8.25 g/l).

Parameter	4.5	6	7.5	9	10.5
P1	0.00+0.00	0.00+0.00	1.16+1.16	1.83+1.83	6.83+6.83
P2	0.00+0.00	0.22+0.22	1.00+1.00	0.00+0.00	2.20+0.41
P3	0.00+0.00	0.00+0.00	1.99+1.67	2.77+1.35	3.88+2.31
P4	0.00+0.00	0.00+0.00	0.22+0.22	0.00+0.00	0.00+0.00
A1	0.00+0.00	1.67+1.67	0.00+0.00	0.66+0.33	18.00+7.75
A2	0.00+0.00	1.67+1.67	0.00+0.00	2.33+2.33	8.50+6.17
B1	0.00+0.00	0.00+0.00	0.00+0.00	0.00+0.00	0.50+0.28
B2	0.00+0.00	0.00+0.00	0.00+0.00	0.00+0.00	1.00+0.57

Table 4. Mean number of: (A) somatic embryos, (B) mature embryos and (c) shoots for susension cultures initiated on different concentrations of 2,4-D (D2B2 = 0.5 mg/l; D3B2 = 1.0 mg/l; D4B2 = 2.0 mg/l) and plated on different media (full concentrations of growth regulator = GR, $^1/_4$ growth regulators concentrations = $^1/_4$ GR, BA only = SU3), at different culture age.

Culture age (month)	Parameter	D2B2			D3B2			D4B2			Total GR, $^1/_4$ GR, SU3		
		A	B	C	A	B	C	A	B	C	A	B	C
2.5	GR	0.0^a	18.7^a	0.0^a	1.7^a	9.0^a	0.0^a	2.0^a	7. 5^a	0.0^a	1.2^a	11.7^a	0.0^a
	$^1/_4$ GR	0.7^a	1.7^a	0.0^a	3.7^a	10.7^a	0.0^a	2.0^a	9.3^a	0.0^a	2.1^a	7.2^a	0.00^a
	SU3	2.7^a	3.3^a	0.0^a	1.0^a	1.3^a	1.7^a	0.0^a	15.0^a	10.3^a	1.2^a	6.6^a	4.0^a
	Mean	1.1^a	7.9^a	0.0^a	2.1^a	7.0^a	0.55^a	1.3^a	10.6^a	3.4^a	1.5^a	8.5^a	1.3^a
4	GR	6.2^a	6.4^a	0.0^a	3.6^a	6.6^a	0.0^a	1.7^a	1.5^a	0.0^a	3.8^a	4.8^a	0.0^a
	$^1/_4$GR	1.3^a	2.2^a	0.0^a	1.5^a	6.52^a	0.0^a	1.8^a	2.0^a	0.0^a	1.6^{ab}	3.6^a	0.0^a
	SU3	0.0^a	10.9^a	0.0^a	0.0^a	3.6^a	7.8^a	0.0^a	1.9^a	0.0^a	0.0^b	5.5^a	2.6^a
	Mean	2.5^a	6.5^a	0.0^a	1.7^a	5.6^a	2.6^a	1.2^a	1.8^a	0.0^a	1.8^a	4.6^a	0.9^a
6	GR	2.4^a	15.8^{ab}	4.2^a	2.5^a	17.3^a	0.8^a	0.7^a	3.7^c	0.0^a	1.9^a	12.3^a	1.7^a
	$^1/_4$ GR	0.0^a	3.5^c	3.9^a	0.1^a	4.6^c	6.3^a	0.0^a	6.3^{bc}	0.0^a	0.0^b	4.8^b	3.4^a
	SU3	0.0^a	0.7^c	9.7^a	0.0^a	0.3^c	3.2^a	0.4^a	8.0^{abc}	2.3^a	0.1^b	2.9^b	5.0^a
	Mean	0.5^a	6.65^a	5.9^a	0.9^a	7.4^a	3.4^a	0.4^a	6.0^a	0.8^a	0.7^a	6.7^a	3.4^a

Table 5. Mean comparisons for: number of embryos, number of mature embryos and number of shoots from suspension culture in different culture ages on MS media with different concentrations of potassium phosphate monobasic (P1 = 1.7; P2 = 3.4; P3 = 5.1; P4 = 8.5 g/l), different potassium nitrate concentrations (A1 = 4.75, A2 = 9.5 g/l) or different ammonium nitrate concentration (B1 = 0.25×, B2 = 0.5×).

Culture age (month)	Embryo			Mature embryo			Shoot		
	2	4	6	2	4	6	2	4	6
P1	0.00^{c}	1.00^{a}	0.00^{c}	1.00^{d}	14.27^{b}	19.62^{a}	0.00^{c}	0.00^{c}	0.00^{b}
P2	0.33^{c}	0.22^{b}	0.00^{c}	13.33^{abcd}	41.63^{a}	6.55^{bcd}	0.00^{c}	0.00^{c}	3.17^{b}
P3	0.00^{c}	0.00^{b}	0.00^{c}	29.00^{ab}	18.37^{b}	3.40^{cd}	0.00^{c}	0.00^{c}	11.17^{b}
P4	6.00^{a}	0.00^{b}	0.00^{c}	1.50^{d}	13.73^{b}	4.20^{c}	0.00^{c}	0.00^{c}	0.00^{b}
A1	0.00^{c}	0.00^{b}	0.00^{c}	8.33b^{cd}	21.60^{b}	12.40^{abc}	0.00^{c}	0.00^{c}	1.00^{b}
A2	1.75^{b}	0.00^{b}	0.00^{c}	5.67^{cd}	11.33^{b}	7.13^{bcd}	0.00^{c}	0.00^{c}	0.00^{b}
B1	0.00^{c}	0.00^{b}	0.00^{c}	26.83^{abc}	27.33^{ab}	0.00^{d}	0.00^{c}	0.00^{c}	36.85^{a}
B2	0.00^{c}	0.00^{b}	0.00^{c}	33.83^{a}	29.20^{ab}	16.17^{ab}	0.00^{c}	0.00^{c}	12.08^{b}

Table 6. Mean comparisons for the number of mature embryos and number of shoots germinating after 8 months on MS media supplemented with different types of sugars with different concentrations of potassium phosphate monobasic (P1 = 1.0×; P2 = 2.0×; P3 = 3.0×; P4 = 4.0×), different potassium nitrate concentrations (A1 = 0.25×, A2 = 0.5×) or different ammonium nitrate concentration (B1 = 0.25×, B2 = 0.5×).

Parameter	Mature embryo			Shoot		
	Maltose	Lactose	Mean	Maltose	Lactose	Mean
P1	13.27^{bc}	3.00d^{ef}	8.13^{bc}	0.00^{d}	9.5^{c}	4.75^{cd}
P2	10.33^{cd}	0.00^{f}	5.16^{cd}	5.5^{cd}	0.00^{d}	2.75^{cd}
P3	3.00^{b}	22.11^{a}	12.55^{b}	0.25^{d}	2.83^{cd}	1.54^{cd}
P4	0.00^{d}	1.88^{ef}	0.94^{d}	0.00^{d}	0.00^{d}	0.00^{d}
A1	7.33^{bc}	11.3b^{cd}	9.33^{bc}	0.00^{d}	23.30^{b}	11.65^{b}
A2	26.33^{a}	19.50^{ab}	22.91^{a}	5.66^{cd}	5.00^{cd}	5.33^{c}
B1	2.17^{d}	0.00^{f}	1.08^{d}	34.77^{a}	20.50^{b}	27.63^{a}
B2	0.66^{cd}	7.17^{cdef}	3.91^{cd}	1.66^{d}	4.83^{cd}	3.25^{cd}
Mean	7.88^{a}	8.12^{a}	8.00^{a}	5.98^{a}	8.24^{a}	7.11

after two months (Table 5), while the control (1×) gave the highest value after 4 months (1.0). ANOVA showed significant difference between culture media treatments at 5 and 1% for 2 and 4 months culture periods, respectively.

For mature embryos, although, 0.5× ammonium nitrate gave a higher value for the 2nd, 4th and 6th month (33.8, 29 and 16, respectively), the 2× potassium phosphate monobasic concentration showed the highest value of (41) on the 4th month. Mean values on the 8th month (Table 6) showed that 0.5× potassium nitrate gave the highest mean value of 22.9. Also, data showed that lactose was much more efficient than maltose for the all media used giving, means of 8.12. ANOVA showed significant difference between culture media treatments at 5%, after 2th, 4th, and 6th months of culture, respectively.

For mean shoot numbers, 0.25× ammonium nitrate gave higher value after 6 and 8 months (36.8 and 27.6). Also, lactose was superior to maltose after 8 months (8.24). ANOVA showed significant difference between culture media at both 5 and 1% after 6 months culture period.

DISCUSSION

Cymbopogon schoenanthus, being a wild desert grass, is facing hyper arid climatic conditions. The species is well known in Africa for its folk medicine and pharmaceutical importance. Over collection from the natural habitats is threatening the wild populations to the edge of extinction. The species has not been explored for any agricultural or biotechnology practices that may help alleviate the pressure on the natural populations.

The present work on *C. schoenanthus* subsp. *proximus* reports the regeneration of plants from callus and suspension cultures. The study shows the feasibility of producing whole plants from different types of *in vitro* cultures through somatic embryogenesis. The effect of different 2,4-D, phosphorus and nitrogen concentrations were studied and the concentrations most effective for

somatic embryogenesis and whole plant regeneration was determined. Reports of the tissue culture of different species of the genus C*ymbopogon* started in the early 1980's (Jagadishchandra and Sreenath, 1982, 1986; Mathur et al., 1988a, b, 1989a, b; Yadav et al., 2000; Zheng et al., 2007). Early studies aimed at the *in vitro* cloning of elite selections through axillary shoot cultures in *Cymbopogon martinii, C. nardus*, *C*. *citratus and C*. *jwarancusa*. Callus cultures from different *Cymbopogon* species showed regeneration through organogenesis as in *C. winterianus* (Patnaik et al., 1999), or through somatic embryogenesis as in *C. martinii* and *C*. *citratus* (Mathur et al., 1988a, b, 1989a, b). The presence of 2,4-D in the induction media, followed by its removal, resulted in efficient regeneration, through somatic embryogenesis. A short exposure to NAA or IAA enhanced rooting and acclimatization in the different species *C. winterianus* (Mathur et al., 1989a, b) and *C. flexuosus* (Nayak et al., 1996).

The present study showed that *C. schoenanthus* subsp. *proximus* is similar to other species of the genus *Cymbopogon* in its regeneration requirements and route. The use of seeds as explants for producing callus due to the low induction frequency from other explants tested as in seedling leaf sections. The production of embryogenic callus through the use of 2,4-D followed by its reduction or complete elimination at later stages to enhance embryo germination and subsequent rooting, all are the same in the reported species (Mathur, 2000). In *C. schoenanthus,* embryogenic callus at a frequency of 90 to 100%, containing mature well formed embryos was produced on MS media containing 1.0 and 4.0 mg/l 2,4-D. Suspension cultures required lower concentrations of 2,4-D (1.0 and 2.0 mg/l) for embryo induction than callus cultures. The effect of BA on embryo germination and shoot production from suspension cultures after plating was more pronounced than in callus cultures. The present study also showed that in callus cultures, somatic embryo induction was enhanced by high phosphorus concentration and low concentrations of both NH_4NO_3 and KNO_3. Embryo maturation was highest on low NH_4NO_3, while embryo germination and shoot production was enhanced significantly, using low (0.25× and 0.5×) KNO_3 concentrations. The positive effect of high phosphorus on somatic embryogenesis in suspension cultures was more pronounced since it enhanced significantly both embryo induction and maturation. Low levels of both NH_4NO_3 and KNO_3 (0.5×) was effective for embryo maturation only. Shoot germination from mature embryos produced from suspension cultures were positively affected by mainly low KNO_3 concentrations (0.25×).

The effect of nitrogen concentrations and type has been reported in literature for a wide range of species. In wheat, raising the NH_4NO_3 concentration significantly increased the induction of primary embryos and their transition into shoots (Greer et al., 2009*)*. In *Coriandrum sativum*, Murthy et al. (2008) showed that low NH_4NO_3 and high KNO_3 enhanced embryo maturation and germination. Shankarmurthy and Krishna (2006) showed that high levels of KNO_3 (4.0 to 5.0 g/l) significantly increased the development of somatic embryos in the medicinal plant *Bridelia scandens*. Also, in the different *Coffea species*, *Embelia ribes, Vigna unguiculata* and *Sapindus mukorossi*, high KNO_3 enhanced significantly embryo development and maturation (Samson et al., 2006; Krishna et al., 2004; Ramakrishnan et al., 2005; Sinha et al., 2000).

The effect of high phosphorus concentration on embryo induction and maturation, especially its enhancing effect on embryo maturation and conversion into healthy green shoots, in suspension cultures, of *C. schoenanthus* subsp. *proximus*, have not been reported so far for other species. Studying zygotic embryos of olives*,* Maalej et al. (2006) found that at early developmental stages, all mineral elements were at their highest level particularly phosphorus (P) and sodium (Na). At differentiation and maturation stages, all elements were lower except for P and Na which were relatively higher at maturation stage only.

The present work presents regeneration of *C. schoenanthus* subsp. *proximus,* a medicinally important species that is distributed only in Africa. Regeneration was achieved through somatic embryogenesis from callus and suspension cultures. This will be important for the clonal propagation, genetic improvement and the *ex situ* conservation of the species.

ACKNOWLEDGMENTS

The research was carried out through grant #79 from the Egyptian Academy of Scientific Research and Technology, Plant Biotechnology and Genetic Engineering program, to whom the authors are grateful.

Abbreviations

BA, 6-Benzyl-amino purine (N^6 benzyladenine); **GA_3,** gibberellic acid; **HFM,** hormone free media; **IAA,** indole-3-acetic acid; **MS,** Murashige and Skoog (1962); **NAA,** α-naphthaleneacetic acid; **2,4-D,** 2,4-dicholorophenoxyacetic acid.

REFERENCES

Batanouny KH, Abou T, Shabana M, Soliman F (1999). Wild medicinal plants in Egypt. Palm Press, Cairo, Egypt.

Bhattacharya S, Bandopadhtay TK, Ghosh PD (2009). Somatic embryogenesis in *Cymbopogon pendulus* and evalution of clonal fidelity of regenerants using ISSR marker. Scientia Horticulturae, 123: 505-513.

Boulos L (1983). Medicinal Plants of North Africa. Reference Publication Inc. Michigan, USA. p. 92.

Boulos L (2005). Flora of Egypt, Monocotyledons. Al Hadara publishing, Cairo, Egypt, 4: 336-340.

Dey T, Bhattacharya S, Ghosh PD (2010). Somatic embryogenesis from rhizome explants of *Cymbopogon winterianus*. Biol. Plant, 325-328.
Duncan DB (1955). Multiple ranges of multiple F tests. Biometrics, 11: 1-42.
El-Askary HI, Meselhy MR, Galal AM (2003). Sesquiterpenes from *Cymbopogon proximus*. Molecules, 8: 670-677.
Elgamal MH, Wolff P (1987). A Further contribution to the sesquiterpenoid constituents of *Cymbopogon proximus*. Planta Medica, 53(3): 293-294.
Greer MS, Kovalchukl I, Eudes F (2009). Ammonium nitrate improves direct somatic embryogenesis and biolistic transformation of *Triticum aestivum*. New Biotechnol., 26: 44-52.
Jagadishchandra KS, Sreenath HL (1982). *In vitro* culture and morphogenetic studies in some species of *Cymbopogen* Spreng. (aromatic grass). In: Fujiwara A (ed). Plant tissue culture 1982. Maruzen, Tokyo, pp. 703-704.
Jagadishchandra KS, Sreenath HL (1986). *In vitro* culture of rhizome in *Cymbopogon* Spreng. and *Vetiveria* Bory. In: Reddy GM (Ed.). Proceedings of national symposium on recent advances in plant cell and tissue culture of economically important plants. Osmania University, Hyderabad, India, pp. 199-208.
Khadri A, Serralheiro MLM, Nogueira JMF, Neffati, Smiti S, Aroٍjo MEM (2008). Antioxidant and anti-acetyl cholinesterase activities of essential oils from *Cymbopogon schoenanthus* L. *Spreng.* Determination of chemical composition by GC-mass spectrometry and 13C NMR. Food Chem., 3: 630-637.
Krishna V, Shankarmurthy K, Maruthi KR, Nagaraja YP, Rahiman BA (2004). Somatic embryogenesis and regeneration of plantlets from leaf callus culture of *Embelia ribes*. J Trop. Med. Plants, 5: 95-99.
Locksley HD, Fayez MBE, Radwan AS, Chari VM, Cordell GA, Wagner H (1982). Constituents of local plants XXV, Constitution of the antispasmodic principle of *Cymbopogon proximus*. Planta Medica, 45: 20.
Maalej M, Chaari-Rkhiss A, Drira N (2006). Contribution to the improvement of olive tree somatic embryogenesis by mineral and organic analysis of zygotic embryos. Euphytica, 151: 31-37
Mathur AK, Ahuja PS, Pandey B, Kukreja AK, Mandal S (1988a). Screening and evaluation of somaclonal variations for quantitative and qualitative traits in an aromatic grass *Cymbopogon winterianus* Jowitt. Plant Breed., 101: 321-334.
Mathur AK, Ahuja PS, Pandey B, Kukreja AK, Mathur A (1988b) Development of superior strains of an aromatic grass *Cymbopogon winterianus* Jowitt, through somaclonal variation. In: Proc. International conference on research in plant sciences and its relevance to the future, Delhi University Press, Delhi, India, p. 160.
Mathur AK, Ahuja PS, Pandey B, Kukreja A (1989a). Potential of somaclonal variation in the genetic improvement of aromatic grasses. In: Kukreja AK, Mathur AK, Ahuja PS, Thakur RS (Eds.), tissue culture and biotechnology of medicinal and aromatic plants. Paramount Publishing House, New Delhi, India, pp. 79-90.
Mathur AK, Ahuja PS, Kukreja AK, Pandey B (1989b). Progress in the application of tissue culture for the improvement of aromatic grasses. In: Proc. XIII Annual meeting of plant tissue culture association of India, North-Eastern Hill University, Shillong, India, p. 31.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiol., 15: 473-497.
Murthy HN, Hahn EJ, Paek KY (2008). Recurrent somatic embryogenesis and plant regeneration I *Coriandrum sativum* L. Scientia Horticulturae, 118: 168-171.
Patnaik J, Sahoo S, Debata BK (1999). Somaclonal variation in cell suspension culture-derived regenerates of *Cymbopogon martinii* (Roxb.) Wats. var. *motia*. Plant Breed., 118: 351-354.
Nayak S, Debata BK, Sahoo S (1996). Rapid propagation of lemongrass Cymbopogon flexuosus (Nees) Wats. through somatic embryogenesis *in vitro*. Plant Cell Rep., 15: 367-370.
Ramakrishnan K, Gnanam R, Sivakumar P, Manickam A (2005). *In vitro* somatic embryogenesis from cell suspension cultures of cowpea [*Vigna unguiculata* (L.) Walp]. Plant Cell Rep., 24: 449-461.
Samson NP, Campa C, leGal L, Noirot M, Thomas G, Lokeswari TS, deKochko A (2006). Effect of primary culture medium composition on high frequency somatic embryogenesis in different *Coffea* species. Plant Cell Tissue Org. Cult., 86: 37-45.
Shankarmurthy K, Krishna V (2006). Regeneration of plantlets via somatic embryogenesis from leaf culture of *Bridelia scandens* Willd. Phytomorphology, 56: 101-105.
Sheweita SA, Newairy AA, Mansour HA, Yousef MI (2002). Effect of some hypoglycemic herbs on the activity of phase I and II drug metabolizing enzymes in alloxan-induced diabetic rats. Toxicology, 2: 131-139.
Sinha RK, Majumdar K, Sinha S (2000). Somatic embryogenesis and plantlet regeneration from leaf explants of *Sapindus mukorossi* Gaertn.: a soapnut tree. Curr. Sci., 78: 620-623.
Snedecor GW, Cochran WG (1981). Statistical Methods 7. The Iowa state Univ. Press, Ames, Iowa, U.S.A.
Sreenath HL, Jagadishchandra KS (1982). Clonal propagation of Java citronella (C*ymbopogon winterianus* jowitt) by tissue culture. In: Proc 69 th Sess Indian Science Congr, Abstr. Indian Sci. Congr Assoc, Calcutta, Pp. 95-96.
Sreenath HL, Jagadishchandra KS (1987). Nuclear cytology of Khavi grass (*Cymbopogon jwarancusa* (Jones) Schult. under *in vivo* and *in vitro* system. Cell Chr. Res. 10: 93-99
Tapia A, Georgirev M, Bley T (2007). Free radical scavengers from *Cymbopogon citratus* (D.C.) Stapf. Plants cultivated in bioreactor of the temporary immersion principle. Z. Naturforsch. Sect. C J. Biosci., 62: 447-457.
Yadav U, Mathur AK, Sangwan NS (2000). *In vitro* calli-clone regeneration to enhance qualitative variability in essential oil of a wild *Cymbopogon* species. In: Kumar S, Kukreja AK, Dwivedi S, Singh AK (Eds.). Proc. of the national seminar on research and development in aromatic plants: Current trends in biology, uses, production and marketing of essential oils. J. Med. Pl. Sci. (Suppl. 1B), CIMAP Publication, Lucknow, India, p. 485-487.
Yentema O, Alioune O, Dorosso SA (2007). Chemical composition and physical characteristics of essential oil of *Cymbopogon schoenanthus* (L.) Spring of Burkina Faso. J. Appl. Sci., 7: 503-506.
Zheng Z, Zhou S, Zhang J, Qing GJ, Xu GZ, Guilin X (2007). Study on the *in vitro* culture of African lemongrass. Acta Agric. Shanghai, 23: 61-64.