Micropropagation through *In Vitro* Tillering from Seed Cultures of the Medicinal Plant *Cymbopogon schoenanthus* subsp. *proximus*

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ABSTRACT— ***Cymbopogon schoenanthus subsp. proximus is a wild aromatic herb that produces essential oils and terpenoids of pharmaceutical, antimicrobial and antioxidant properties. It is becoming endangered as a result of over collection for its multi-uses. Effect of different factors on axillary multiplication and growth from seed cultures in vitro were studied. Those included benzyl adenine concentrations, different sugar types, sugar alcohol types and concentrations and different silver nitrate concentrations. Among different concentrations of benzyl adenine used, 7 mg/l produced the highest number of shoots (27.0±4.5 shoots/explant), fresh weight and dry weight. Maltose, glucose and sucrose gave significantly higher shoot numbers and fresh weight than fructose, lactose and galactose. Addition of sorbitol in concentrations 2.25% in combination with 2.25% of sucrose significantly increased shoot numbers (35.5 ± 5.8 shoots/explant), while different concentrations of mannitol either did not affect or negatively affected biomass production. Silver nitrate at 2 mg/l improved shoots proliferation (33.4±3.7 shoot/explant), fresh weight and dry weights. For rooting, different concentrations of three auxin types used did not induce any rooting. Well develop strong roots were produced only on media containing 6% of glucose or sucrose. Humic acid significantly increased root growth. This study established an efficient, reproducible and rapid protocol for in vitro mass production of the species that can be used in folk medicine, in drug production, and also for ex-situ conservation.***

Keywords— Organogenesis, sugars, silver nitrate, sugar alcohols, humic acid

1. INTRODUCTION

Cymbopogon schoenanthus subspecies *proximus* is a wild aromatic grass that grows in the desert south of Egypt, northern of Sudan and subtropical Africa [1]. The plant has been used for decades in traditional medicine as anti-diabetic, diuretic, bronchodilator, and antispasmodic [2, 3]. Currently the wild plant extract is used as a safe medicine (Proximol*) for expulsion of ureteric stones, uric acid dissolvent and as urinary antiseptic. Chemically, the plant is characterized by the presence of mono and sesquiterpenoids, mainly proximadiol which possesses the bioactive compound used for the propulsion renal calculi [4, 5]. As a result of over collection of this species for traditional medicine and drug production purposes, the wild plant population has declined in its natural habitat. No cultivation attempt or conservation program of this species is yet reported.

The efficiency of plant micro-propagation system and organ growth and development depends on many factors, such as growth regulators and carbon source in the culture media [6, 7]. Carbon source and its concentration affect in shoot proliferation of many plants [8-10]. Humic acids are organic molecules present naturally in the soil and are often known as the most important component of a healthy fertile soil [11]. The effect of humic acid on plant growth and development were reported in many studies [12-14].

The *in vitro* propagation of genus *Cymbopog*on was carried out via somatic embryogenesis and shoot tip culture [15-17]. Initial research on *C. schoenanthus* subsp. *proximus* propagation was achieved through somatic embryogenesis [18, 19] due to its potential for large scale production using bioreactor and also to use somatic embryogenic cultures/tissues as transformation target. Although somatic embryo induction was efficient, both maturation and germination were slow. Direct organogenesis from seed culture was found to contain the bioactive compound proximadiol [20]. Therefore, there is a need for rapid *in vitro* propagation of this important species.

In the present work our objective was to study a number of physiological factors that may affect efficient and reproducible shoot axillary multiplication (tillering) and rooting from mature seed culture. We examined the effect of benzyl adenine, different sugar types, sugar alcohols, silver nitrate and humic acid. We report here a successful micropropagation system using quick and efficient *in vitro* axillary multiplication, followed by strong rooting and acclimatization to produce plants that can be used for drug production, *ex-situ* conservation and habitat restoration to maintain the species genetic pool in the wild. The high demand for the species and its low propagation rate under desert dry climatic conditions makes biotechnological improvement of this species an important target.

2. MATERIALS AND METHODS

2.1 Plant material

Mature *C. shoenanthus* subsp. *proximus* inflorescences were collected from Aswan Botanical Garden, Egypt during spring of the years 2012-2015.

2.2 Chemicals:

Chemicals were purchased either from Sigma Aldrich (St. Louis, MO, USA) or Phyto-Technology (USA).

2.3 Seed sterilization:

Healthy seeds were collected from mature inflorescence one day before culturing. Seeds collected on cheese cloth were washed under tap water for 15 min followed by 5 min wash in distilled water. Surface sterilization was carried out by stirring the seeds for 1 min in 95 % ethanol, 20 min in 20 % Clorox solution. Surface sterilized seeds were then washed 3 times 5 min each, with sterile distilled water under aseptic conditions.

2.4 Shoot multiplication:

Surface sterilized seeds were cultured on Murashige and Skoog medium (1962) with Gamborg's vitamins (1968) solidified with 0.2% phytagel (MSB5); pH was adjusted to 5.8 before autoclaving.

A) Effect of BA concentrations: Sterilized seeds were cultured on media with different concentrations from benzyl adenine BA (0.0, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mg/l) in combination with 0.05 mg/l NAA (α - Naphthalene acetic acid).

For all the following experiments BA concentration (7 mg/l) and NAA concentration 0.05 mg/l were constant in all media.

B) Effect of sugar types: To test the effect of carbon source type, 6 sugars were tested separately (fructose, galactose, glucose, maltose, lactose and sucrose) at 3 % concentration.

C) Effect of sugar alcohol concentrations: Different concentrations of sorbitol and mannitol were tested separately (0.75, 1.5, 2.25, 3.0, 3.75, and 4.5 %). Each concentration from sorbitol or mannitol was combined with the same concentrations of sucrose.

D) Effect of silver nitrate concentrations: (0.5, 1.0, 1.5, 2.0 mg/l) of silver nitrate were tested.

For all experiments: Two liquid media additions (10 ml in each) were carried out. First addition was carried out after 4 weeks from seed culture with MSB5 and 0.2 mg/l BA, sugar concentration was 2 % (the same sugar type used in the solid medium). Second addition was carried out after 6 weeks from initial seed culture with media containing the same sugar concentration as first liquid media and lacking growth regulators.

2.5 Rooting experiments

A) Effect of auxin type and concentrations: Shoots longer than 2 cm produced from experiment A in shoot multiplication were separated onto single shoots or groups of three shoots and transferred to rooting MSB5 medium supplemented with different concentrations of indole-3-acetic acid (IAA) , or indole-3-butyric acid (IBA), or naphthalene acetic acid (NAA) at 0.05, 0.1, 0.5 mg/l.

B) Effect of humic acid (HA) concentrations: Seeds were cultured on MSB5 medium containing 3% sucrose. Different concentrations of HA (0.0, 0.75, 1.5, 3.0, 6.0, 9.0, 15.0 mg /l) were added. After 4 weeks, 10 ml hormone free media with 2 % sucrose were added to the control and 10 ml with 2% sucrose and 1.5 mg/l HA were added to all humic acid treatments. After 6 weeks from culture initiation another 10 ml containing 2 % sucrose were added to each magenta.

C) Effect of sugar and NAA concentrations: Seeds were cultured on MSB5 medium supplemented with two concentrations of either sucrose or glucose (3.0 and 6.0 %). After 4 weeks from seed culture 10 ml with 2% sugar

supplemented with 0.2 mg /l BA were added to each magenta. After 7 weeks, 10 ml with 2 % sugar with different NAA concentrations (0.0, 0.01, 0.1, 0.2 mg/l) were added to each magenta.

2.6 Culture incubation conditions: All cultures were incubated at 25 ºC under cool white fluorescent light (3000 lux) for 16/8 h light/dark photoperiod.

2.7 Acclimatization: Healthy plantlets were carefully cleaned by removing excess media by washing with tap water, transplanted into pots containing garden or sandy soil and covered with polyethylene bags to maintain humidity. Plantlets were watered daily with tap water and incubated at 25°C under white fluorescent light for one week. Humidity was gradually decreased. After 2 months plants were transferred to the greenhouse under ambient condition with programmed watering.

2.6 Data collection and statistical analysis: For each experiment, data were collected from each seed explants on number of shoots, fresh weight and dry weight. For each treatment, data were collected from 16-24 seed explants cultured in 4-6 magenta boxes with 4 seeds in each. Data were analyzed using Minitab 17 software by one way ANOVA. Where between treatment differences are significant, treatment means were compared by Fisher least significant difference (LSD) Method with 95 % Confidence level.

3. RESULTS AND DISCUSSION

3.1 Shoot multiplication

A) Effect of BA concentrations: Different BA concentrations were highly significant on shoot multiplication, fresh weight and dry weight with P value = 0.00 (Fig. 1). Our results indicate that, 7.0 mg / l BA was significantly superior in shoot multiplication with average number of (27.0 ± 4.5) shoots / explant and (3.9 ± 0.99, 1.5 ± 0.7 g) fresh and dry weights, respectively. Similar effects of BA in the range (5-10 mg/l) were found to be efficient in shoot proliferation in different plants such as wheat, rice and banana [21-23]. Additionally, combining NAA with BA enhances shoots proliferation with improved rooting of species as in *Clitoria ternatea* and *Prunella vulgaris* [24, 25].

B) Effect of different sugar types: Different sugars (3 % concentrations) showed significant effect in shoot numbers and fresh weight only with P value 0.015. Dry weight values were non-significant. Maltose produced the highest shoot number 20.5 ± 4.9 shoots / explant and was significantly better than fructose, lactose and galactose (Fig. 2). The effect of glucose and sucrose was non-significant compared to maltose in number of shoots. Lactose and galactose produced the lowest values of fresh weight 1.75 ± 0.08 g and 0.005 ± 0.001 g, respectively, while maltose and glucose produced highest values 2.35 ± 0.5 g, 2.12 ± 0.48 g, respectively. Effect of sugar types on shoot multiplication differs from species to species, for example glucose was the best sugar in case of *Fagussyl vatica* and *F. orientalis* plants while fructose produced negative effect on the growth of shoots [26]. On the other hand, fructose was superior to sucrose; glucose and maltose in *Physocar pusopulifolius* shoot multiplication [10] and better than sucrose in shoot multiplication of two types from *Ficus carica* cultivars [27] Galactose gave the lowest shoot numbers compared to sucrose, glucose, and fructose in *Dahlia* shoot multiplication while glucose produces the maximum number of shoots [28].

C) Effect of sugar alcohols: The effect of different concentrations of sorbitol and mannitol on number of shoots, fresh and dry weights is shown in table 1. Both 1.5 and 2.25 % sorbitol in combination with the same concentration of sucrose were significantly favorable for shoot multiplication by producing an average of 32.8 ± 10.7 and 35.5 ± 5.8 shoot/explants, respectively. For both fresh and dry weight control as well as 1.5 and 2.25 % sorbitol in combination with the same concentration of sucrose were found to be better than all other concentrations of sorbitol and mannitol. Our results agree with [29] who reported that the presence of sorbitol as a carbon source in 'San Castrese' *Prunus armeniaca* L. culture media enhanced shoot proliferation and elongation. Also sorbitol increased the proliferation of peach rootstocks [30, 31]. Sorbitol is usually considered as a main storage carbon source in some plant species like apple [32]. Also, it plays a role as osmoregulator. [33] Reported that the importance of osmoregulation in organ initiation on tobacco callus shoot formation. Use of different concentrations of mannitol in our *C. schoenanthus* cultures decreased the number of shoots. Similar results were reported by [34] on *Plumbago rosea* L. and by [35]on *Piper aduncum* and *P. hispidinervum*. Mannitol is considered as a poorly metabolized sugar in plants which is known to decrease the osmotic potential and repress lateral root [36, 37].

D) Effect of silver nitrate concentrations: Different concentrations of silver nitrate produced significant effect on shoots proliferation, fresh weight and dry weight (Fig 3). Silver nitrate with 2 mg/l increased shoot numbers, fresh and dry weights (33.4 ± 3.7 shoot/explant, 4.6 ± 0.67 g, and 1.4 ± 0.45 g) respectively compared to the control. The positive effect of silver nitrate on shoot multiplication was reported on different plant species [38-40]. Plant tissues grown *in vitro* produce ethylene. Accumulation of ethylene in the tissues negatively affects growth, development and lead to early

senesce in some plants as in Papaya [41]. Silver nitrate considered as an ethylene inhibitor, which when supplied in the culture media prevents the negative effect of ethylene on *in vitro* germinated plants [39, 42].

3.2 Rooting and Acclimatization

A) Effect of different type and auxin concentration: Different concentrations of IAA, IBA, and NAA used showed no effect on rooting.

B) Effect of HA concentrations: Effect of different concentrations from HA (0.75 to15 mg / l) on root length, fresh and dry weight is shown in table 2. Both concentrations 6.0, 9.0 mg/l of HA produced significantly higher values in root dry weight (0.04 ± 0.01, 0.02 ± 0.004 g respectively). Although 6 mg / l produced high mean value in root length (9.2 ± 2.5) cm, all treatment means differences were not statistically significant. However, several studies reported the positive effect of humic acid in root development, root length and root growth [13, 43, 44].

C) Effect of sugar concentrations in combination with different NAA concentrations: High concentration of glucose and sucrose increased root number and length compared to the control (3 %) with p value = 0.0, 0.01 respectively. Different concentrations of NAA didn't affect adventitious root development (Table 3). ANOVA for different NAA concentrations and their interaction with sugar type and sugar concentration were all non-significant. Using 6 % glucose with 0.0 NAA produced higher root number (18.4 ± 5.0) followed by 6 % sucrose combination with 0.2 mg / l NAA (12.4 ± 0.07). Higher mean value of root length (9.8 ± 5.3) was recorded with 6 % sucrose with 0.01 mg/l NAA. Fresh weights produced higher values with 6 % sucrose in combination with 0.1, 0.2 mg/l NAA (0.51 ± 0.1, 0.34 ± 0.005 g) respectively. Glucose at 6 % without NAA or with low NAA concentration 0.01 mg / l were superior regarding to dry weight values.

Successful acclimatization with 100% frequency was achieved from all rooted plantlets on 6% sugar concentration.

Our results agree with those reported in Peonies plants [45] where high concentration of glucose (9 %) in the absence of growth regulator increased root number. Similarly, higher concentration (90 and 145 mM) of sucrose increased root number and root length in Carob tree and apple plants [46]. Also, osmotic stress has been found to increase plant regeneration frequency in some cereals [47, 48].

Sugars play an important role in maize root development, since it used as source of energy, cell wall component and compatible solute [49].

Table 1 Effect of sugar alcohol type and concentrations (%) on mean number of shoots (Shoot No), fresh weight (Fw) and dry weight (Dw).

Sucrose concentration (%)	Sugar alcohol		Shoot No	Fw (g)	Dw (g)
	Mannitol concentration (%)	Sorbitol concentration (%)			
3.0	0.0	0.0	15.8 ± 7.7 bc	2.3 ± 1.6 ab	1.5 ± 1.10 a
0.75	0.75	0.0	2.3 ± 1.4 bc	0.2 ± 0.1 c	0.09 ± 0.06 c
1.5	1.5	0.0	8.8 ± 4.8 bc	1.0 ± 0.6 bc	0.25 ± 0.14 bc
2.25	2.25	0.0	1.6 ± 0.5 c	0.1 ± 0.07 c	0.08 ± 0.04 c
3.0	3.0	0.0	5.0 ± 4.2 bc	0.4 ± 0.3 c	0.2 ± 0.19 bc
3.75	3.75	0.0	3.3 ± 1.9 bc	0.4 ± 0.1 c	0.2 ± 0.11 bc
4.5	4.5	0.0	14.5 ± 8.3 bc	0.3 ± 0.1 c	0.16 ± 0.08 bc
0.75	0.0	0.75	1.0 ± 0.4 c	0.1 ± 0.04 c	0.08 ± 0.01 c
1.5	0.0	1.5	32.8 ± 10.7 a	3.0 ± 0.9 a	1.1 ± 0.12 ab
2.25	0.0	2.25	35.5 ± 5.8 a	2.4 ± 0.7 ab	1.1 ± 0.27 ab
3.0	0.0	3.0	16.6 ± 4.9 b	1.0 ± 0.5 bc	0.47 ±0.19 bc
3.75	0.0	3.75	2.0 ± 1.0 bc	0.4 ± 0.2 c	0.06 ± 0.03 c
4.5	0.0	4.5	0.9 ± 0.5 c	0.1 ± 0.04 c	0.04 ± 0.02 c

*In each column means followed by the same letter are not significantly different at p< 0.05 level according to Fisher test.

Table 2 Effect of humic acid concentrations on root length (Root L), root fresh weight (Fw) and root dry weight (Dw)

Humic acid concentration (mg / l)	Root L. (cm)	Fw (g)	Dw (g)
0.0	4.8 ± 1.5 a	0.04 ± 0.01 a	0.008 ± 0.0004 bc
0.75	4.2 ± 1.5 ab	0.23 ± 0.20 a	0.02 ± 0.005 bc
1.5	5.6 ± 1.5 a	0.05 ± 0.02 a	0.02 ±0.005 b
3.0	4.4 ± 1.6 ab	0.03 ± 0.01 a	0.01 ± 0.004bc
6.0	9.2 ± 2.5 a	0.08 ± 0.04 a	0.04 ± 0.01a
9.0	6.4 ± 1.4 a	0.05 ± 0.01 a	0.02 ± 0.004ab
12.0	6.8 ± 3.3 a	0.04 ± 0.01 a	0.01 ± 0.003bc
15.0	6.5 ± 2.0 a	0.03 ± 0.01 a	0.01 ± 0.002bc

*In each column means followed by the same letter are not significantly different at p< 0.05 level according to Fisher test.

Table 3 Effect of sucrose and glucose concentrations (%) in combination with NAA concentrations (mg/l) on average root number (Root No.), root length (Root L.), fresh weight (Fw) and dry weight of roots (Dw).

Sucrose conc. (%)	Glucose conc. (%)	NAA conc. (mg/l)	Root No.	Root L. (cm)	Fw (g)	Dw(g)
3.0	0.0	0.0	0.5 ± 0.4 de	0.7 ± 0.50 cde	0.01 ± 0.005 c	0.001 ± 0.0002 e
		0.01	2.3 ± 1.4 de	2.0 ± 1.20 bcde	0.06 ± 0.03 bc	0.01 ± 0.0010 de
		0.1	1.1 ± 0.9 de	3.0 ± 1.90 bcde	0.05 ± 0.04 bc	0.01 ± 0.0010 de
		0.2	0.1 ± 0.08 e	0.2 ± 0.10 e	0.01 ± 0.002 c	0.004 ± 0.0006 e
6.0	0.0	0.0	9.2 ± 3.6 bc	5.6 ± 1.30 ab	0.14 ± 0.050 abc	0.03 ± 0.0010 bc
		0.01	5.5 ± 1.5 cd	9.8 ± 5.30 a	0.12 ± 0.035 bc	0.02 ± 0.0010 cd
		0.1	8.8 ± 0.4 bc	2.1 ± 2.10 bcde	0.51 ± 0.010 a	0.03 ± 0.0010 bc
		0.2	12.4 ± 0.1 b	5.3 ± 0.10 abcd	0.34 ± 0.005 ab	0.03 ± 0.0100 bc
0.0	3.0	0.0	0.0 ± 0.0 e	0.0 ± 0.00 e	0.00 ± 0.000 c	0.0 ± 0.0000 e
		0.01	0.8 ± 0.7 de	0.9 ± 0.90 bcde	0.03 ± 0.025 c	0.02 ± 0.0040 ce
		0.1	0.5 ± 0.5 de	0.4 ± 0.30 de	0.01 ±0.007 c	0.002 ± 0.0001 e
		0.2	0.5 ± 0.3 de	1.5 ± 1.00 bcde	0.02 ± 0.010 c	0.002 ± 0.0001 e
0.0	6.0	0.0	18.4 ± 5.0 a	3.6 ± 1.10 bcde	0.25 ± 0.200 abc	0.06 ± 0.0100 a
		0.01	11.3 ± 0.1 b	5.5 ± 1.10 abc	0.21 ± 0.005 abc	0.04 ± 0.0050 b
		0.1	6.0 ± 0.4 bc	2.1 ± 0.07 bcde	0.05 ± 0.025 bc	0.01 ± 0.0010 de
		0.2	10.3 ± 2.9 bc	3.3 ± 1.40 bcde	0.13 ± 0.050 bc	0.02 ± 0.0010 cd

*In each column means followed by the same letter are not significantly different at p< 0.05 level according to Fisher test.

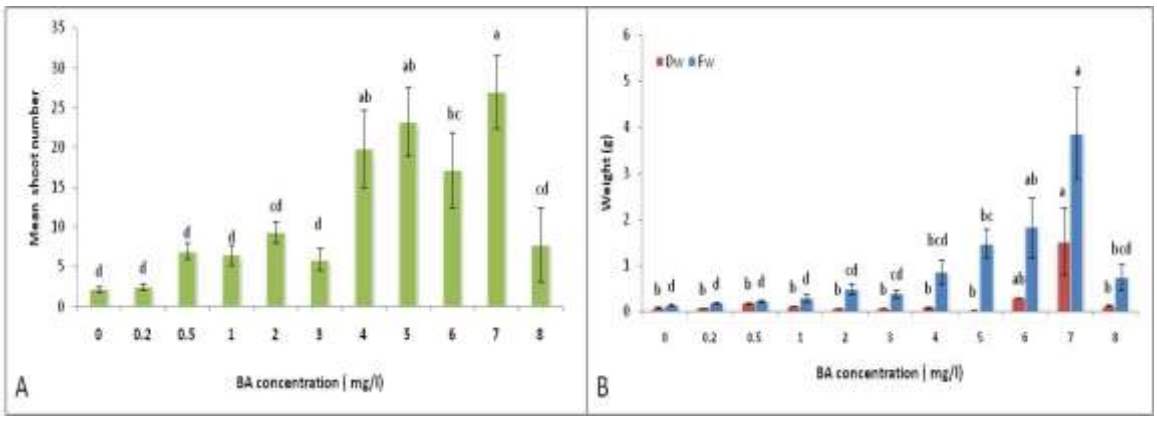

Figure 1 Effect of different BA concentrations (mg/l) on mean number of shoots, fresh weight (Fw) and dry weight of shoots (Dw).

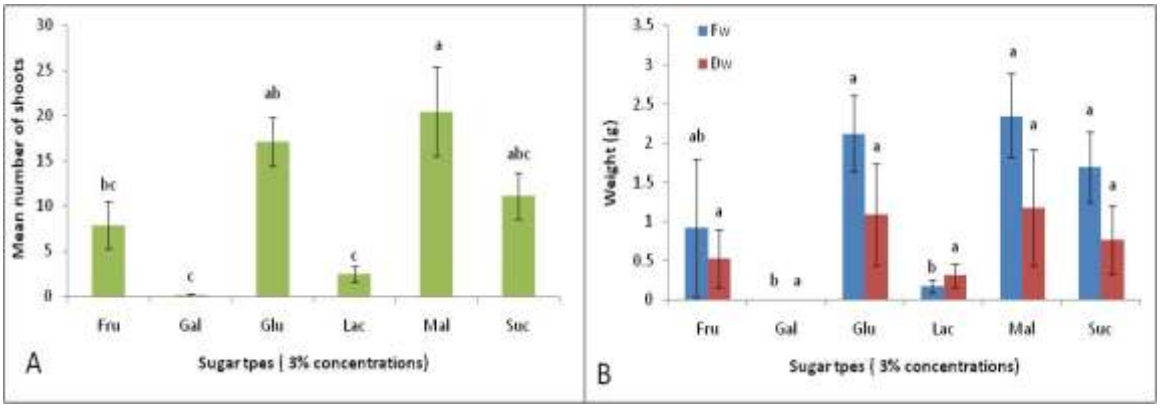

Figure 2 Effect of sugar types (Fru = Fructose, Gal = Galactose, Glu = Glucose, Lac = Lactose, Mal = Maltose and Suc = Sucrose) on mean number of shoots (A), fresh weight (F. Wt) and dry weight (D. Wt) of shoots (B).

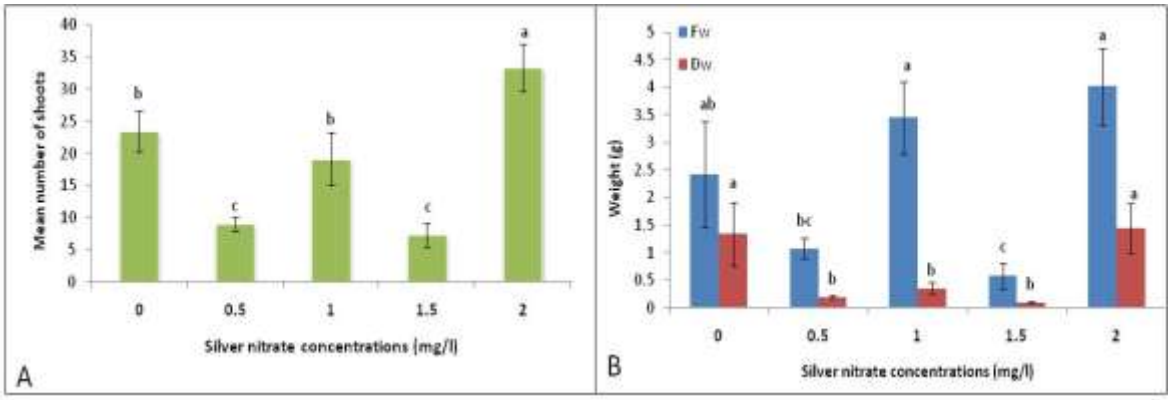

Figure 3 Effect of silver nitrate concentrations (mg/l) on: Mean number of shoots (A), fresh weight (Fw) and dry weight (Dw) of shoots (B).

*In each figure means followed by the same letter are not significantly different at $p< 0.05$ level according to Fisher test.

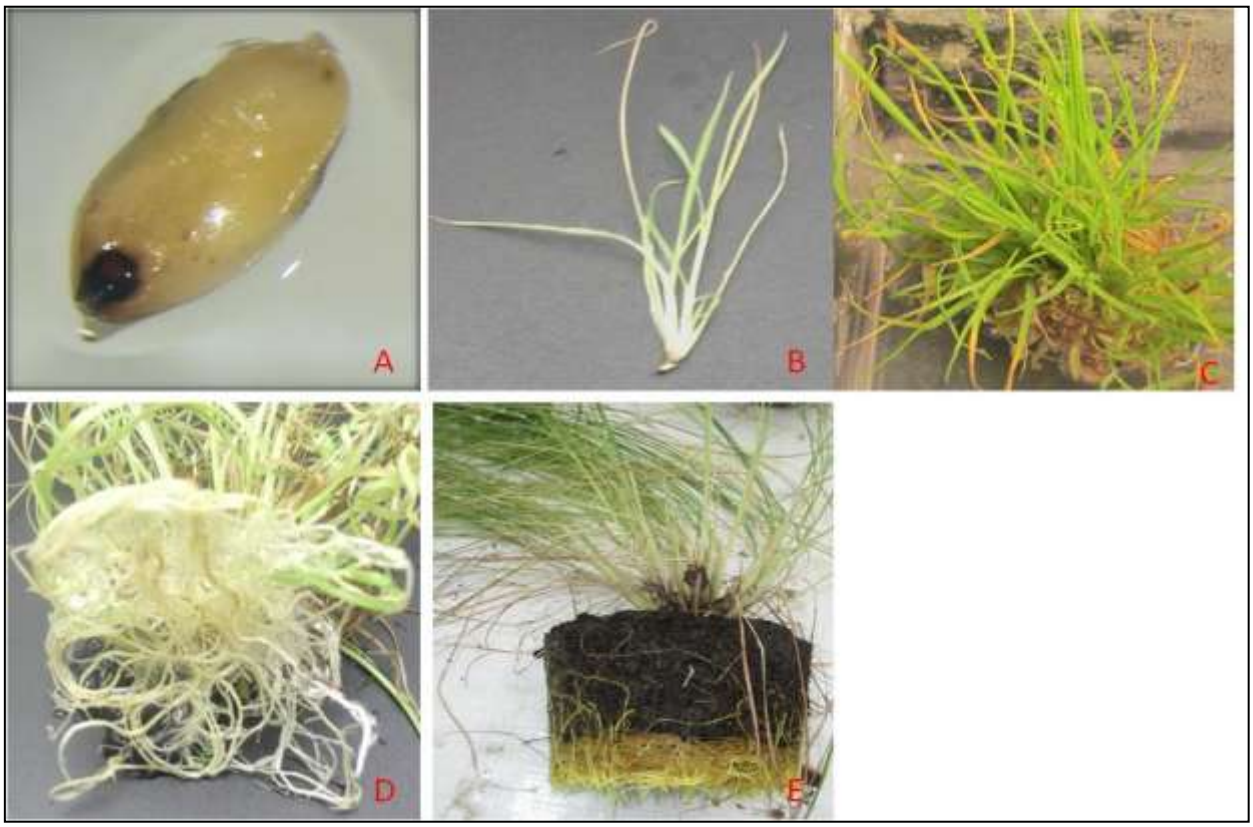

Figure 4 Direct organogenesis from *C. shoenanthus* A) Seeds under light microscope; B) Shoot after 1 week from culturing; C) High axillary multiplication (tillering) D) Rooted plants on 6% sucrose; E) Acclimatized plant.

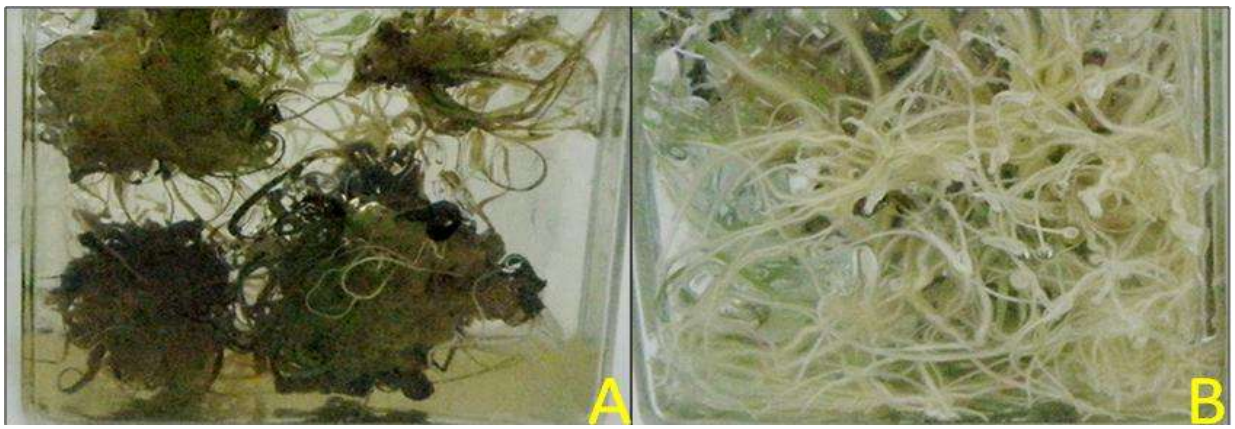

Figure 5 Effect of sugar concentration on rooting: A) roots produced in 3 % sucrose concentrations, B) roots produced on 6% sucrose concentration.

4. CONCLUSION

Cymbopogon schoenanthus subsp. *proximus* heavily used in both folk medicine and as a source of diuretic antispasmodic drug proximol* is under heavy collection pressure from its natural habitat. This will lead to habitat destruction especially when coupled with harsh climatic conditions. The present work presents an efficient/short span simple protocol for *in vitro* tillering followed by rooting and acclimatization that produce strong propagaules that can be used both for *ex-situ* conservation, habitat restoration and also for bioactive compound production.

The system applies the physiological factors for the control of growth *in vitro*. Growth regulators, sugars, sugar alcohol, silver nitrate and humic acid in the culture media, were all tested and were found to affect the rate of axillary multiplication, shoot growth, adventitious root induction and root growth. Sugar concentrations and sugar alcohol were significant in both shoot multiplication and root induction. Humic acid significantly increased growth of roots. The system was found to contain the bioactive compound proximadiol [20]. This will be useful for studies of factors controlling bioactive compounds *in vitro* and for selection of superior clones of economic importance. Strong propagules in a short time can be provided for *ex-situ* conservation, horticulture practices and for habitat restoration.

5. ACKNOWLEGEMENT

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