Nig. J. Biotech. Vol. 37(1): 159-168 (June 2020)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: https://dx.doi.org/10.4314/njb.v37i1.17

NIGERIAN JOURNAL OF BIOTECHNOLOGY

# Effects Of Different Sterilization Regimes & Growth Regulators On Micropropagation Of Female Date Palm (*Phoenix dactylifera* L.)

***[1]Emoghene, B.O., [2]Idu, M., [1]Eke, C.R. and [1]Asemota, O.**

[1]Physiology Division, Nigerian Institute for Oil palm Research (NIFOR), PMB 1030, Benin City, Nigeria.

[2]Plant Biology and Biotechnology Department, University of Benin, Benin City, Nigeria.

**Abstract**

**The success of *in vitro* culture techniques is always hampered by microbial contamination. The present study was carried out to develop an efficient protocol for date palm explants sterilization for successful somatic embryos induction and plantlets formation of some date palm varieties. The shoot tips were treated with different sterilizing agents at different concentrations and durations of exposure. The use of ethanol (70%), sodium hypochlorite (3.5% & 70%) and mercuric chloride (0.2%) with or without addition of Tween-20 had different effects on decontamination of the date palm explants. The percentage of explants contaminated with bacteria for sterilants 1, 2 and 4 was 18.8%, 6.3% and 6.3% respectively while 25%, 37.5%, 31.25% and 6.25% were contaminated with fungi for sterilants 1, 2, 3 and 4 respectively. Under the conditions used, a combination of antioxidants (Citric and Ascorbic acids at 100mg/l), 0.2% mercuric chloride and 3.5% sodium hypochlorite solution with 3 drops/100ml of Tween-20 helped in the reduction of chlorosis, contamination and die-back in the shoot tip explants. The explants were further cultured in appropriate media for callus initiation and subsequent somatic embryo induction. Optimal embryogenic callus was obtained from the shoot explant of sterilant number 4 which had the minimal contamination and die-back of all the cultures. After 3 subcultures, the somatic embryos formed were multiplied for shoot development. From this study, we established that the use of appropriate surface sterilant at suitable concentration and duration of exposure of date palm explant to it is indispensable for maximum responses of *in vitro* cultures.**

**Keywords:** Date palm, Microbial contamination, Sterilizing agents, *in vitro*, Somatic embryos
***Corresponding author:** emoghenebo21@gmail.com, +234-8023555479.

## Introduction

Date palm (Phoenix dactylifera L.) grows well in the hot sahelian zone of Northern Nigeria, where it is an important agricultural commodity (Asemota and Eke, 2005). The high demand for female date palms on the local market in Nigeria requires expansion of increased production. High-yielding elite genotypes are needed for cultivation, but cannot be met by conventional breeding alone. Micropropagation through tissue culture is one of the most widely accepted tools for the large scale production of genetically improved plant materials (George and Sherrington, 1984). Date palm explants are exposed to microbial infection at all stages of tissue culture. Contamination might come with the explants or occurs during the propagation procedures. Different explanations have been proposed about the causes of microbial contamination of date palm tissue culture (Abass, 2013). One explanation is the method used to sterilize the explants, tool and equipment. Improper

methods and insufficient amounts of disinfectants are the most common problems in tissue culture. Another problem is external contamination of the explants which comes from contaminated tools, equipment and workers in the preparation and culturing of media (Abass, 2013). The composition of the tissue culture medium is a good source of nutrients for microbial contaminants, with all the essential requirements to support their growth and development (Abass, 2013).

Culture initiation is the first and most critical stage in micropropagation (Daud *et al.,* 2012; Vujovic *et al.,* 2012). The major adverse effects of microbial contamination on date palm tissue culture are degradation and browning of the infected tissues caused by the release of substances into the medium, such as degrading enzymes (cellula se, phenol oxidase and others); microbes such as viruses, bacteria, yeast and fungi are found on the surface and inside the plant body, as well as toxins (Abass, 2013). Microbial contamination leads to waste of time, effort and material and contributes to severe economic losses (Hameed and Abass, 2006; Omamor *et al.,* 2007). There is competition between the microbes and the plants for nutrients.

The success of plant tissue culture protocol depends largely on sterilization of the explants. Selection of sterilizing agents and time of exposure is also critical because the living material should not lose their biological activity, and only contaminants should be eliminated during sterilization (Tiwari *et al.,* 2012). Utilizing a proper and precise sterilization can be vital to save time and effort. The different sterilants used on explant materials for *in vitro* multiplication affect tissue responses. Therefore, suitable concentration, exposure duration of the explants to the various sterilants and the sequence of using these sterilants have to be standardized to minimize explants injury and achieve better survival (Rady *et al.,* 2018). The present study was undertaken to unveil the effects of different types of sterilizing agents by varying their concentrations and time of exposure on date palm explants for induction of somatic embryos and plantlets formation.

**Plate 1a:** Excised offshoots under laminar flow condition before commencement of the sterilization procedure

**Plate 1b:** Explant sterilization using sterilants at different concentrations and timing before inoculation into the media.

## Materials and Methods

*Sample collection*

Explant materials (offshoot of 2 to 3 years) were excised from selected special palms of female dates with good fruit quality at the date palm experimental substation of the Nigerian Institute for Oil Palm Research (NIFOR), Dutse, Jigawa State.

*Explants Preparation*

The method of Khan and Bibi (2012) was used for explants preparation. This was then treated with different sterilizing agents at different concentrations and timing (Plate 1a and1b).

*Sterilization Procedure*

*Sterilant 1:* The explant was kept in 70% ethanol (EtOH) for 10 min, rinsed 2-3 times in

sterilized distilled water. It was then immersed in 10 % sodium hypochlorite (NaOCl) solution mixed with three drops/100ml of Tween-20 for 15 min and rinsed thrice in sterile distilled water.

*Sterilant 2:* The explant was kept in 70% ethanol (EtOH) for 10 min, rinsed 2-3 times in sterile distilled water, then kept in 10% Sodium hypochlorite (NaOCl) solution mixed with 3 drops/100ml of Tween-20 for 15min and rinsed 3 times in sterile distilled water. It was finally kept in 0.1% mercuric chloride for 5min and then rinsed thrice in sterile distilled water.

*Sterilant 3:* The explant was sprayed with 70% ethanol (EtOH) and left for 5 min after which it was further reduced and kept in 0.2% Mercuric Chloride ($HgCl_2$) for 10min, rinsed 3 times in sterile distilled water. It was then dipped into 3.5% sodium hypochlorite (NaOCl) solution containing 3drops/100ml of Tween-20 for 20min and thereafter rinsed 3 times in sterile distilled water.

*Sterilant 4:* **The** explant was kept in an antioxidant (Citric and Ascorbic acids at 100mg/l) for 5min, rinsed once in sterile distilled water. Then it was immersed in 0.2% Mercuric Chloride ($HgCl_2$) and placed on an orbital shaker for 10min, rinsed 3 times in sterile distilled water. It was finally kept in 3.5% sodium hypochlorite (NaOCl) solution containing 3 drops/100ml of Tween-20 for 20 min and then rinsed 3 times in sterile distilled water.

*Culture Medium*

Full strength of Murashige and Skoog (MS, 1962) culture medium was used for this experiment. The following supplements were incorporated in the culture medium 0.02% glutamine, 0.0125% myo-inositol, 0.0075% citric acid and ascorbic acid 3.0% sucrose and 0.8% agar. Combination of growth regulators (Table 1) with 1.5 g/l activated charcoal were tested for callus initiation. The pH was adjusted to 5.8 using potassium hydroxide (KOH) after addition of all compounds. Further trimming of the shoot tips was done in order to remove the upper and lower regions exposed to sterilants (Plate 2). It was then sectioned longitudinally and inoculated into the media (Plate 3). The cultures were incubated in the dark at 27±1°C with a proper and regular record for contamination and browning taken for 30 days. Only one factor was studied in this research because of limited explant materials. The surviving explants with the different sterilizing agents were subcultured every 4 weeks into the same media composition to evaluate embryogenic callus and somatic embryos for plantlets formation.

**Table 1:** Media containing auxins and cytokinin for explants initiation.

| Medium No. | Growth regulators (mg/l) | |
|---|---|---|
| 1 | Basal MS | |
| 2 | NAA (100) | + 2-iP(3) |
| 3 | 2,4-D(100) | + 2-iP(3) |

KEY: NAA = 1-naphthalenacetic acid; 2, 4-D = 2, 4-dichlorophenoxyacetic acid and 2-iP = 6-dimethylallylamino purine

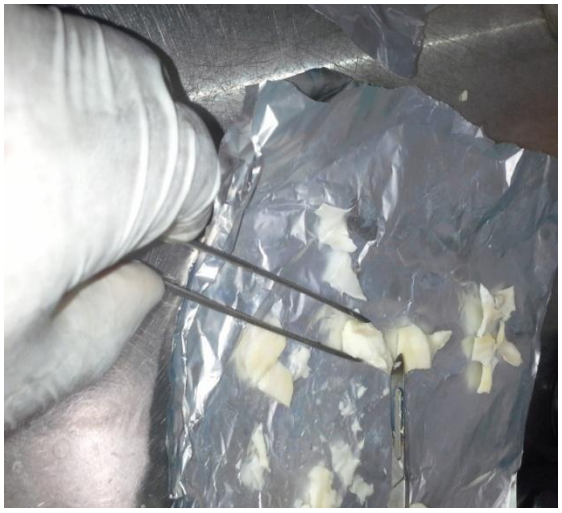

Plate 2: Trimmed Shoot tip explants after sterilization to expose the meristem.

Plate 3: Inoculation of shoot tips immediately after dissection into culture media containing growth regulators at different concentrations

**Results**

*Explants Sterilization*

The result of the various effects of sterilizing agents on the contamination and colour change (browning) of the explants is presented in Figure 1. Percentage contamination was considerably higher in the explants sterilized with sterilants 1 and 2 than those that received sterilants 3 and 4 involving the use of Mercuric chloride and antioxidant solutions. The percentages of the explants contaminated with bacteria (Plate 4a and 4b ) for sterilants 1, 2 and 4 were 18.8, 6.3 and 6.3 % respectively, while 25, 37.5, 31.25 and 6.25% were contaminated with fungi (Plate 4c) for sterilants 1, 2, 3 and 4, respectively.

The explants sterilized using sterilant 4 proved better than those of steilants 1, 2 and 3. In contrast to contamination, the percentage chlorotic colour or necrosis developed by the explants was highest in the second sterilized explants with about 25% of the explants turning chlorotic after 4 weeks in the cultures. The fourth sterilized explants showed the least colour change with only 6.25% of them turning chlorotic with the highest percentage of survived cultures (Figure 1). Based on the result of this experiment, the explants were consistently sterilized in subsequent studies using the fourth method of sterilization which involves treatments with antioxidants (Citric and Ascorbic acids at 100 mg/l), 0.2% Mercuric chloride and 3.5% Sodium hypochlorite solution containing 3 drops/100ml of Tween-20.

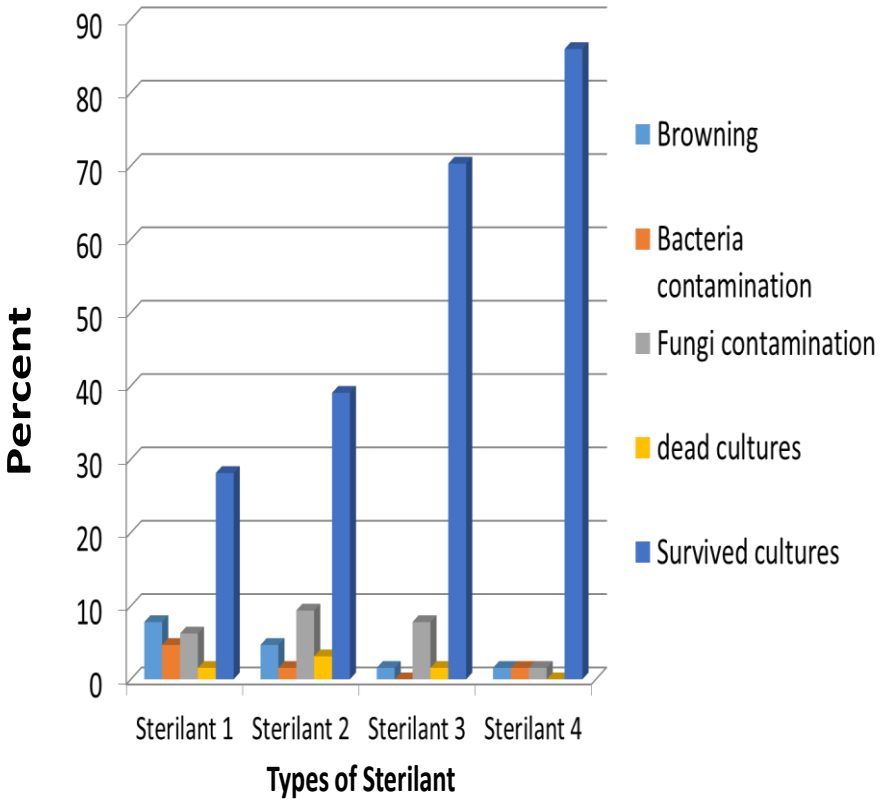


**Figure 1**: Effect of sterilizing agents on explants of date palm after 4 weeks in culture

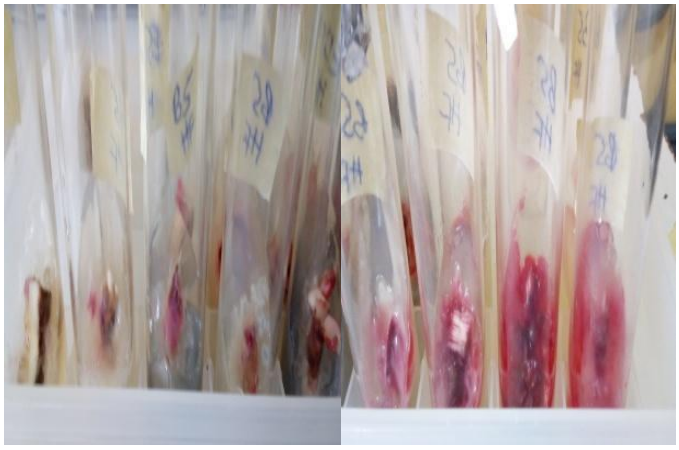

Plate 4a and 4b: Bacterial contaminations on the explant cultures after a few weeks of inoculation.

Plate 4c: fungi contamination on explant culture after few weeks from the day of inoculation.

*Callus and Somatic embryos induction*

Embryogenic callus and somatic embryos were observed after 4 to 12 weeks in the initiation media from the remaining surviving cultured explants. A maximum response was observed from the cultures of sterilant number 4 due to minimal contamination and die-back. The culture medium supplemented with 2, 4-D (100mg/l) and 2-iP (3mg/l) gave the highest callus and somatic embryogenic response (Plate 5a and 5b).

Plate 5a and 5b: Embryogenic callus and somatic embryogenic response after three months of subculture into fresh media

*Callus multiplication and somatic embryos development*

The callus obtained multiplied in a medium containing MS basal salts supplemented with growth regulators (NAA, kinetin and BAP) (Plate 6). The somatic embryos (Plate 7) that resulted from the embryogenic calli were isolated and cultured (Plate 8) in a suitable medium. Some of these embryos multiplied slowly, others (Plate 9) proliferated almost indefinitely with the mature ones concurrently developing shoots . The shoots were isolated for multiplication and elongation (Plate 10 & 11) and later rooted using MS medium supplemented with NAA (0.1mg/L) (Plate12)

Plate 6: Embryogenic callus responses from the initiated explant subcultured into multiplication medium for further multiplication and development.

Plate 7: Somatic embryos development from the embryogenic calli after few weeks of subculture into fresh media .

Plate 8: Harvested somatic embryo from callus culture.

Plate 9: Embryos multiplication with shoot development after two subcultures into fresh medium.

Plate 10: Shoot development from matured somatic embryos.

Plates 11: Shoots elongation stage showing well developed plantlets.

Plate 12: Rooted plantlets under pre-acclimatization process.

*Plant Acclimatization*

The regenerated plantlets were transferred to a mixture of peat moss and soil at equal volumes (v/v) and then kept in a plastic bag (Plate 13) under green house conditions and wetted with 10% MS once every week. Plantlets were sprayed with 2.5% benlate solution every 2 to 3 days to prevent crown and leaf rot. After 8-12 months, well acclimatized and hardened plants (Plate 14) were transferred to the open field for further monitoring.

Plate 13: Plant acclimatization stage under controlled humidity and temperature.

Plate 14: Hardening plant in nursery after successful acclimatization process.

**Discussion**

The present investigation was carried out to optimize sterilization protocol on date palm shoot tip for induction of somatic embryos and plantlets formation *in vitro.* Under the conditions used for the sterilization of the explants, a combination of antioxidants (Citric and Ascorbic acids at 100mg/l), 0.2% mercuric chloride and 3.5% sodium hypochlorite solution containing 3 drops/100ml of Tween-20 proved to be the best of all the four sterilizing agents (Figure 1) as it greatly reduced chlorosis, contamination and die-back in the explants.

The use of ethanol, sodium hypochlorite and mercuric chloride had different effects on decontamination of the explants. The ineffectiveness and harmful effects of ethanol on decontamination of plant tissues have been observed by other researchers like Hammond *et al*. (2014) who recorded 100% contamination when 70% ethanol was used alone in the sterilization of sweet potato explants. Similarly, Sen *et al*. (2013), studying seed germination of *Achyranthes aspera in vitro* reported 100% contamination when ethanol alone was used for sterilization and that ethanol was more harmful to the explants. Furthermore, Osterc *et al*. (2004) also reported low germination rates when 70% ethanol was included in the sterilization of *Pinus sp.* There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ (Sen *et al* 2013). However, exposure to $HgCl_2$ may have negative effects on the survival rates of explants. A long period of exposure to $HgCl_2$ leads to browning and death of the explants (Sen *et al* 2013). Estrela *et al*. (2003) reported that the effectiveness of sodium hypochlorite (2%) improved with increasing time of exposure. Acheampong *et al.* (2015) in their study reported that the duration of exposure of pineapple explants to sodium hypochlorite improved sterilization; however, exposure for long durations beyond 20 minutes were detrimental to the explants. Sodium hypochlorite has been reported to be very effective against many types of bacteria (Tiwari *et al.,* 2012). Very small concentrations can reduce bacterial populations (Nakagawara *et al.* 1998).

Sterilization is effective with the combination of different sterilants at reasonable concentrations. From the economical perspective a cheap, simple and effective sterilization protocol is needed (Tiwari *et al.,* 2012). Mamun *et al*., 2004 in their work on micropropagation of some important sugarcane varieties reported that the use of low concentrations of $HgCl_2$ (0.1%) gave satisfactory results. The explant was unresponsive and turned black when treated with higher concentrations (0.2, 0.3 and 0.4 % $HgCl_2$) for 8-10 min.

In this study, the auxin-cytokinin interactions enhanced the formation of somatic embryos from the embryogenic calli for plantlets formation. The culture medium supplemented with 2, 4-D (100mg/l) and 2-ip (3mg/l) gave the

highest calli and somatic embryos. Most researchers suggest 2, 4-dichlorophenoxy acetic acid (2, 4-D) as the most effective auxin to induce embryogenic callus (Al-Khayri and Naik, 2017). Asemota *et al., (*2010) reported the use of 100mg/l NAA or 2, 4-D and 3mg/l 2-ip to induce embryogenic callus from the meristem and leaf of date palm explants.

The somatic embryos from the embryogenic calli were transferred to growth regulator-enriched media for germination and multiplication (Plates 8 and 9). The cultures with shoots development were separated into multiplication and elongation media supplemented with NAA, BAP and 2-ip. Eke *et al*. (2005) also obtained several plantlets on a growth regulator- enriched medium. The role of plant growth regulator in increasing the shoot length of date palm was also reported by Khierallah and Bader (2007).

## Conclusion

The success of plant tissue culture depends on sterilization of explants and therefore selection of sterilizing agents and time of exposure are very critical. This study identified the combination of sterilizing agents at an appropriate concentration, combination and duration of exposure for reduction of contamination and tissue browning for successful micro propagation of date palm.

## References

Abass M.H. (2013). Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi tissue culture laboratories. *Emir. J. Food Agric.* 25(11):875-882.

Acheampong, S., Galyuon, I.K.A., and Asare, A.T. (2015). Effect of Sterilization protocols, benzylaminopurine and type of explants on growth initiation of pineapple (*Ananas comosus* (L.) Merr.) Cultures. *Journal of Basic and Applied Sciences,* 1**(3)**, 50-65.

Al-Khayri, J.M. and Naik, M. (2017). Date palm micropropagation: Advances and applications. *Cienc. Agrotec*. 41:4

Asemota, O. and Eke, C. R. (2005). Direct shoot development from date palm in culture. *Agriculture* No. 3094.

Asemota, O., Eke, C. R., Emoghene, B. O., Aisueni, N. O., Adetunji, O. A., Gidado, R. M. and Solomon, B. O. (2010). Investigation of somatic embryogenesis for in vitro cultures of date palm (*Phoenix dactylifera* L.). *Acta Hort*. **882**: 225 – 231.

Daud, N.H., Jayaraman, S., and Mohamed, R. (2012). Methods paper: An improved surface sterilization technique for introducing leaf, nodal and seed explants of *Aquilaria malaccensis* from field sources into tissue culture. *As. Pac. J. Mol. Biol.andBiotech.***2**: 55-58.

Eke, C.R., Akomeah, P. and Asemota, O. (2005). Somatic embryogenesis of Date palm (*Phoenix dactylifera* L.) from apical meristem tissues from "Zebia" and "Loko" landraces. *Afr. J. Biotechnol*. 4(**3**): 244 – 246.

Estrela, C., Ribeiro, R. G., Estrela, C. R., Pecora J. D., and Sousa-Neto, M.D. (2003).Antimicrobial effect of 2% sodium hypochlorite and 2% chlorhe xidine tested by different methods. *Braz Dent J* 14 (**1**): 58-62.

George, E. F. and Sherrington, P. D. (1984). Plant Propagation by Tissue Culture; handbook and Directory of Commercial Laboratories. Exegenices Ltd, Basingstoke, England. Pages: 709.

Hameed, M. A. and Abass M.H. (2006). Study of cytological changes associated with contaminated date palm (*Phoenix dactylifera* L.) tissue cultures with fungi. *Basra Res. J.* **32**: 1-27.

Hammond, R., Buah, J. N., Asare, P. A. and Acheampong, S. (2014). Optimizing sterilization condition for initiation of sweet potato (*Ipomoea batatas*) culture *in vitro.Asian Journal of Biotechnology.* ISSN1996-0700/D01: 10. 39231 ajbkir.

Khan, S. and Bibi, T. (2012). Direct shoot regeneration system for date palm (*Phoenix dactylifera* L.)cv. Dhakki as a means of micropropagation. *Pak. J. Bot.* 44(6): 1965-1971.

Khierallah, H. S. and Bader, S. M. (2007). Micropropagation of date palm (*Phoenix dactylifera* L.) var. Maktom through direct organogenesis *Acta Horticulture*. **736**: 213-224.

Mamum, M. A., Sikdar, M.B.H., Paul, D. K., Rahman, M.M. and Islam, M. R. (2004). *In*

*vitro* micropropagation of some important sugarcane varieties of Bangladesh. *Asian. J. Plant Sci.,* **3**: 666-669.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantrum***15**: 473-497.

Nakagawara, S., Goto, T., Nara, M., Ozawa Y., Holta, K. and Arata, Y. (1998). Spectroscopic characterization and pH dependence of bactericidal activity of the aqueous chlorine solution. *Anal. Sci.* **14**:691-698.

Omarmor, I. B., Asemota, A. O., Eke, C.R. and Eziashi, E.I. (2007).Fungi contaminants of the Oil Palm tissue culture in Nigerian Institute for Oil Palm Research (NIFOR). *Afr. J. Agric. Res.***2**:534-537.

Osterc, G., Luthar, Z. And Stampar, F. (2004). The importance of the sterilization procedure for producing vigorous cherry plants (*Prunus sp.*) *in vitro*. Acta agriculturae Slovenica, 83-1. 45-51.

Rady, M.R., Saker, M.M and Matter, M.A. (2018). *In Vitro* Culture, transformation and genetic fidelity of Milk Thistle. *J. Genet. Eng. Biotechnol.* 16(2):563-572.

Sen, M.K., Jamal, M.A.H.M. and Nasrin S. (2013). Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured *in vitro. Environmental and Experimental Biology,* 11:119-123.

Tiwari, S., Arya, A. and Kumar, S. (2012). Standardizing Sterilization Protocol and Establishment of Callus culture of Sugarcane for Enhanced Plant Regeneration *in vitro*. *Research Journal of Botany,* **7:1**-7.

Vujovic, T., Ruzic, D.J. and Cerovic, R. (2012). *In vitro* shoot multiplication as influenced by repeated subculturing of shoots of contemporary fruit rootstocks. *Hort. Sci.* (Prague), **39**: 101-107.