Research Journal of BioTechnology

Vol. 8 (11) November 2013

Journal is indexed by Chemical Abstracts, SCOPUS and SCIE having Impact Factor 0.294

Micropropagation and chloroplast isolation from *in vitro* of *Aloe Vera* plants

Hoang T. K. H.
Department of Biology, Faculty of Sciences, Hue University, 77 Nguyen Hue, Hue, VIETNAM
hkhong@husc.edu.vn

Abstract
The plant, Aloe vera L., belongs to the Liliaceae family and is well-known as an important pharmaceutical plant that is used for investigating and creating medicinal and cosmetic compounds. Notably, this specie displays all of the physiological features of CAM (Crassualacean acid metabolism) photosynthesis along with exhibiting net nocturnal CO_2 uptake. Among CAM plants, A. vera is one of numerous phosphoenolpyruvate carboxykinase (PCK)-CAM plants which contain significant activities of PCK with lower levels of malic enzyme (ME). Generally, A. vera requires the operation of malate dehydrogenase (MDH) to convert malate to oxaloacetate (OAA). OAA is further converted to phosphoenolpyruvate (PEP) and CO_2 by cytosol PCK.

Although the micropropagation of A. vera process has been well-studied, very little attention has been given to the role of the A. vera chloroplasts from in vitro plants during the CAM photosynthesis. In doing this research, we produced the in vitro plants of A. vera by creating a tissue culture and then we used the leaves of in vitro plants to isolate pure, intact chloroplasts by the use of Percoll density gradient centrifugation.

Keywords: *Aloe vera*, Chloroplast, *in vitro* plants, Micropropagation, Percoll.

Introduction

Aloe vera is commonly known as a valuable herbal plant which has an important role in cosmetic formulations, food supplements as well as having antioxidant, antimicrobial, anti-inflammatory, anticancer and antimalarial properties[10,25]. This essential soothing gel obtained from *A. vera* leaves makes for an excellent treatment of wounds including burns and other skin disorders, by providing a protective coat over the affected area and thus speeding up the rate of healing while reducing the risk of infection[5]. A significant element that *A. vera* contains, is the very impressive medicinal compound such as barbaloin, which along with some other phytoconstituents, is chiefly responsible for its pharmacological activity.

It is also used clinically in the treatment of elevated blood glucose levels, the prevention of the sun's UV ray damage and in countering bacterial and fungal infections[11,25]. Given the numerous compounds formulated by the pharmaceutical and cosmetic industries, a great demand for *A. vera* exists. However, due to the limitations on the cultivation of *A. vera* leaves, these industries are left nowadays with an inadequate supply. This is due to the process of their natural propagation; *A. vera* plants use auxillary shoots which in turn lead to the slow multiplication of these plants.

Furthermore, undertaking large-scale cultivation of *A. vera* is not feasible because of the inherent nature of male sterility which provides a barrier to their rapid propagation[24]. Fortunately, today a tissue culture technique has been developed which can solve these problems. This technique is a useful biotechnology tool for both the multiplication of the original herbal plant species as well as analyzing their secondary metabolites, pharmaceutical usage etc. Several reports have been brought to the attention of the rapid *in vitro* propagation of *A. vera* with subsequent success in producing these plants[2,4,14,15].

One of the remarkable features of CAM photosynthesis metabolism as evidenced in CAM plants entails the diurnal closing of the stomata of *A. vera* plants which occurs up to ten times a day. This activity reduces the potential of the leaves experiencing evaporation. At night, the stomata open in order to absorb CO_2 and accumulate CO_2 in the form of malic acid. The metabolism of malic acid during the day helps the plants discharge CO_2 which the plants in turn use in conjunction with water and light to affect its photosynthesis process[17]. This is a fundamental difference between CAM plants (where CO_2 becomes fixed during the dark phase of the photosynthesis) and those of C3 and C4 plants. This unique mechanism of CAM plants enables them to adapt and survive in the extremes of climate found in arid lands.

Let us consider *A. vera* which as previously noted is a CAM plant. As it relates to the nocturnal phase, the stomata of *A. vera* plants, by opening, commences a biochemical process. This process is operated by phospho*enol*pyruvic carboxylase (PEPC) which catalyzes the first reaction of photosynthesis in the leaves of *A. vera*. In this instance, all of the CO_2 that enters the leaves of *A. vera* plants is converted into sugars; thus, CO_2 assimilation by this enzyme is very efficient[25].

Indeed, the *A. vera* plants demonstrate another important efficiency, namely an ability to function well even in situations of water restriction. This is because, again, by the efficient synthesis of sugar, these sugars in turn make such osmotic adjustments possible[17]. This mechanism helps *A. vera* to grow and develop under drought condition better

than C3 and C4 plants.

In fact, *A. vera* prefers light (sunny weather), requires well-drained soil, can grow in nutritionally poor soil and is able to adapt and survive under the stressing conditions of arid and semiarid environments/zones[3]. Given the current global climate changes, they are suitable for cultivation for the aforementioned reasons (i.e. stomata closing during heat of the day, CO_2 availability for photosynthesis in endogenous acid pools). The purpose of this study is to develop a large amount of *in vitro* plants rapidly and then use these plants as a material to optimize a protocol for isolating pure, intact of *A. vera* chloroplasts. Likewise, it is hoped that these intact chloroplasts will prove also useful for further analysis of the physiological photosynthesis and CAM characteristics of *A. vera in vitro* plants.

Material and Methods

Plant materials: Plants of *A. vera* were propagated vegetatively and grown in plastic pots filled with 1:1 mixture of soil and vermiculite. The plants were transferred to a greenhouse with heater that provided natural light and temperature. Water was supplied every three days and compound fertilizer was supplied every two weeks. After 6 months, the plants were selected and kept in a separated place to serve as the experimental materials. The shoot tip explants were selected from these healthy, disease-free plants and used for *in vitro* tissue cultures.

Micropropagation of *A. vera*: The collected samples were transported to the laboratory, rinsed thoroughly in running water and washed with sterile distilled water. Then, they were rinsed with 70% (v/v) ethanol for 2 min and subsequently the surfaces of the leaves were sterilized with a 0.2% (w/v) $HgCl_2$ solution for 10 min. After rinsing with sterile distilled water four times, the explants (0.4-0.5 cm in length) were used to initiate the cultures.

In this research, we chose the optimum tissue culture conditions for rapid micropropagation of *A. vera* according to the procedure of Truong et al[29] with specific modifications. For multiple shoots generation, the explants were cultured on a basal MS medium[13], supplemented with 3% (w/v) sucrose, 20% (v/v) coconut water (CW), 1 gl^{-1} charcoal and 1 mgl^{-1} BAP for shoot induction. The pH of the medium was adjusted to 5.8 before gelling with 8 gl^{-1} agar and autoclaving at 121^{o}C for 20 min. The cultures were maintained at 25 ± 1^{o}C and exposed to 12 h of light at an intensity of 3000-4000 lux.

The formed multiple shoots that emerged from the initiation period of culture were separated aseptically into the individual shoots. Part of these individual shoots (0.5 cm x 0.5 cm) were subcultured on fresh MS medium supplemented with 3% (w/v) sucrose, 20% (v/v) CW, 8 gl^{-1} agar, 1 gl^{-1} charcoal, 1,5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA for shoot multiplication which increased their number. *In vitro* shoot explants (4-5 cm in length) derived from these shoot multiplication cultures were inoculated onto MS medium supplemented with 3% (w/v) sucrose, 20% (v/v) CW, 8 mgl^{-1} agar, 1 gl^{-1} charcoal and 2 mgl^{-1} NAA for root induction.

Plantlets with well–developed shoots (5-8 cm in length) with roots (3-4 cm in length) were removed from the flasks and washed thoroughly in running tap water. They were then transferred to the plastic pots containing soil and sand (1:1) and placed in a greenhouse for acclimatization for two weeks before being planted in the natural condition. The plants were watered twice a week and harvested for chloroplast isolation after three months in the greenhouse.
In order to obtain information about the photosynthetic capabilities of the *A. vera* plant, we used some of these plants to check the amount of water and protein content as well as the concentration of chlorophyll pigments and photosynthetic intensity in leaf extracts.

Leaf samples (second and third leaf pair, numbered from the shoot tip) were taken after 3 hours exposure to light. After collection, the leaves were cleaned and dried. About 2 g (fresh weight) of the leaves were sliced into 0.3 cm thick strips, then immediately frozen in liquid N_2. These samples were subsequently ground up using a mortar and pestle in a 4 mL of extraction medium [50 mM Hepes-KOH (pH 7.6) 8, 8 mM $MgCl_2$, 1 mM EDTA-KOH (pH 7.6), 5 mM DTT, 0.2% (w/v) BSA and 0.02% (w/v) Triton X-100], supplemented with 0.2 g sea sand and 40 mg of PVP-40. After filtration through three layers of sterile Miracloth, part of the homogenate was taken for determination of chlorophyll pigments by the Arnon method[6].

The other part of the homogenate was centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant was desalted by passage through a Sephadex G-25 column equilibrated with the suspending buffer [400 mM sucrose, 0.1% BSA and 40 mM HEPES-KOH (pH 7.4)] and used immediately for determination of any enzyme activity. The enzymes were assayed spectrophotometrically in 1 ml reaction mixtures at 25°C as described by Kondo et al.[17]

Water content in the leaves was determined following their total desiccation. Protein contents were determined with the Bio-Rad protein assay kit according to Bradford[8] using BSA as the standard. Photosynthesis intensity was determined at 9 am by use of a photosynthetic meter (Ciras-2 Portable Photosynthesis System, Licor-USA).

Isolation of chloroplasts from *A. vera in vitro* plants: Intact chloroplasts were isolated and purified from developed leaves of the *in vitro* three month old plants on Percoll gradients using techniques developed by Lang et al[18] with some modifications. For the preparation of intact chloroplasts, the 2nd and 3rd leaf pair (numbered from the shoot tip) of these *in vitro* plants were washed with sterile distilled water and then excess water was drained off.

Approximately 20 g of leaves were used for each experiment. The main part of the leaves was separated out of the non-chlorophyll parenchyma cells and sliced into 0.3 cm thick strips, then homogenized with 80 ml of ice-cold isolation buffer [consisting of 350 mM manitol, 0.1% (w/v) bovine serum albumin (BSA), 1% (w/v) PVP-40, 5 mM $MgCl_2$, 5 mM EDTA-KOH (pH 7.6), 1 mM dithiothreitol (DTT) and 50 mM HEPES-KOH (pH 7.6)] in a Waring blender for 60 seconds with rather strong stirring. After filtration through four layers of sterile Miracloth, the homogenate was centrifuged at 1,500 g for 10 min.

The pellets were resuspended in approximately 10 ml of wash buffer [consisting of 300 mM mannitol, 5 mM $MgCl_2$, 5 mM EDTA-KOH (pH 7.6) and 50 mM HEPES-KOH (pH 7.6)] with the careful use of a very small paint brush. The resuspended chloroplasts were centrifuged at 1,500 g for 5 min. The pellets were resuspended in 2 ml of wash buffer loaded onto a three-step Percoll gradient of 80% (10 ml), 40% (5 ml) and 30% (5 ml) which were prepared with the Percoll buffer containing 300 mM sucrose, 300 mM mannitol, 5 mM EDTA-KOH (pH 7.6), 50 mM HEPES-KOH (pH 7.6). After adding the chloroplasts, the three-step Percoll gradients were centrifuged at 18,000g for 20 min at 4^oC.

After centrifugation, the broken chloroplasts gravitated to the top, forming a large green band toward the top of the Percoll layer, whereas the intact chloroplasts form a sediment-like lower green in the centrifuge tube. The intact chloroplasts were then carefully removed from the gradient using a Pasteur pipette. The chloroplasts were resuspended in 20 ml of wash buffer and collected by centrifuging at 15,000 g for 20 min and repeat this step one more time. Finally, the pellets were resuspended in 1 ml^{-1} of storage solution (grinding buffer but without BSA and PVP-40).

For functional assays, it should be noted that all of these steps were performed at 4^oC and their intact chloroplasts were used as early as possible to prevent loss of protein and enzyme activity.

Enzyme assays: The assays of enzyme activities were carried out on both leaves and intact chloroplasts of *in vitro A. vera* three month old plants. For the enzyme assay, the Percoll-purified chloroplast were filtered at room temperature on a column of Sephadex G-25 equilibrated with the suspending buffer [400 mM sucrose, 0.1% BSA, 40 mM HEPES-KOH (pH 7.4) and 0.02% (w/v) Triton X-100] and then the chloroplasts were collected for measuring the enzyme activities. The enzymes activities were assayed spectrophotometrically in 1 ml reaction mixtures at 25°C.

Cytochrome c oxidase (COX, EC 1.9.3.1) and phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) and initial ribulose 1,5bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) were measured in both the Percoll-purified chloroplast and leaf extracts according to Møller and Palmer[22], Shaheen et al[27] and Du et al.[13]

Results and Discussion

Among CAM plants *Aloe* is one of the special materials which many researchers have used for *in vitro* tissue culture and rapid micropropagation. The results obtained from their studies have shown that *in vitro* culture of *Aloe* is very difficult for both callus induction and plant regeneration. In contrast, micropropagation of the meristems shoots has proved to be easily achieved[26]. Up to now, very little work has been conducted on the callus culture of the Aloe species[1] while many studies have been performed on *A. vera* micropropagation, using meristems[26], shoot tips and axillary buds[21], underground stems, seeds[1], young inflorescences[30] and leaf explants[20] for the explants.

Recently, most of the research has focused on using shoot tips as the main suitable explants for Aloe *in vitro* propagation[1,9,12,19,29]. In the present study, we also used shoot tip explants of *A. vera* as material for rapid production of this plant. The results of our study demonstrate that the use of MS medium culture (pH 5.8) containing 3% (w/v) sucrose, 20% (v/v) CW, 1 gl^{-1} charcoal, 8 gl^{-1} agar and 1.5 mgl^{-1} BAP provides the best result in achieving shoot proliferation from *A. vera* shoot tip explants (Fig. 1).

Fig. 1: Shoot tip explants of *A. vera* showing proliferation after 4 weeks of culture on MS medium supplemented with 3 % (w/v) sucrose, 20 % (v/v) CW, 8 gl^{-1} agar, 1 gl^{-1} charcoal and BAP 1,5 mgl^{-1}.

After culturing for two weeks on this MS medium, most of the samples showed signs of proliferation; new buds appeared and then developed into multiple shoots by the 4th week of culture (Fig. 1). The average number of shoots in this culture medium was 6.4±1.5. No calluses were created from the cultured explants during the beginning of this period; one hundred percent of the shoots grew up well besides.

To increase the potential number of shoots, the new shoot tips and nodal stem segments were excised separately into pieces from the proliferating multiple shoots and further cultured on the same MS medium (above), except for the

supplements of 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA for shoot multiplication. The results showed that all of these cultured samples were regenerated and developed into multiple shoots. These multiple shoots were healthy and grew up within 3 weeks (Fig. 2a) and 5 weeks (Fig. 2b) on this cultured medium.

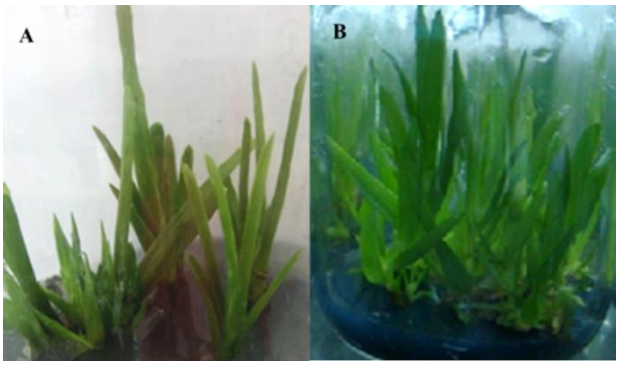

Fig. 2: Multiplication shoots formation from (a) *in vitro* shoot tips after 3 weeks and (b) *in vitro* nodal stem segments after 5 weeks culture on MS medium. Other conditions are shown in fig.1.

The regenerated *in vitro* shoots from each of the multiple shoots survived to form good roots (Fig. 3) and most of the plantlets developed rapidly on the cultured of MS medium supplemented with 3% (w/v) sucrose, 20% (v/v) CW, 8 gl^{-1} agar, 1 gl^{-1} charcoal and 2 mgl^{-1} NAA (Fig. 4).

The results of this research showed that most of the *in vitro* plantlets were successfully acclimatized using a potting mixture containing soil and sand in 1:1 (v/v) ratio and placed in greenhouse for 2 weeks. These plantlets continued to thrive after transferring them to the shade house which was maintained at a natural temperature, humidity and light (Fig. 5).

Generally, the micropropagation of *A. vera* was successfully conducted in this research and the capacity for the survival and development of the *in vitro* plants were quite high. This capacity provided for the production of a significant number of *A. vera in vitro* plants which were further use for chloroplast isolation.

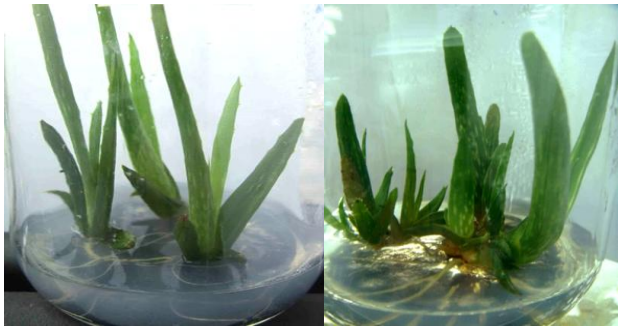

Fig. 3: Root formation of the in vitro plants on MS medium supplemented with 3 % (w/v) sucrose, 20 % (v/v) CW, 8 gl^{-1} agar, 1 gl^{-1} charcoal and 2 mgl^{-1} NAA.

Fig. 4: Growth and development of *A. vera in vitro* plants after 7 weeks culture.

Fig. 5: Regenerated plants of *A. vera* growing in plastic pots after 5 weeks in the greenhouse.

Leaves of *A. vera*possess a great amount of water, the content water of a *A. vera* leaf being about 95.2 % when the leaves are freshly weighed (FW). Likewise, their protein content is about 0.965 mgg^{-1}. Photosynthesis intensity of *in vitro A. vera* plants was about 7.80±0.24 µmol $CO_2m^{-2}s^{-1}$ (Table 1) and this data was rather similar to that of *A. vera* plants in nature[31]. This result indicated that these *in vitroA. vera* plants showed normal photosynthesis capacity. The Chla and Chlb in *A. vera* plants were about 482.5±4.3μgg^{-1} and 279.3±1.4μgg^{-1} respectively. These values were somewhat lower than those of natural *A. vera* plants. However, the Chla/Chlb ratio was similar to that of *A. vera* plants in a natural setting and this ratio reflected CAM characteristics in *in vitro A. vera* plants.

It is well known that chloroplasts are the most important cytoplasmic organelles in the photosynthesis process and they play a significant role in producing the yield in all plants. In addition, the chloroplasts also provide the sites for starch, fatty acid and amino acid metabolism[28]. As has been indicated, isolated organelles are often utilized to evaluate organelle function.

The examination of these numerous physiological parameters, such as those of the enzymatic properties and biosynthetic pathways, requires the analysis of organelles. They have been separated into intact forms from different

kinds of plant species and tissues. In the leaves, most of the cells photosynthetically are active, though the dominant cell organelles are the chloroplasts.

There is often considerable contamination by fragments of broken chloroplasts which lead to problems in isolating and studying other cell fractions of leaves[16]. Although the study of the chloroplasts is a classic one in the field of plant physiology and isolation procedures that yield highly purified and functional chloroplast have been described for many species, including *Arabidopsis thaliana*[27], pea seedlings and Physcomitrella patens[18]. As has been noted previously, the leaves of the *A. vera* plant possess a considerable amount of water. They also have very thick, non-chlorophyll parenchyma cells together with chlorophyll cells and thus remain somewhat more difficult to work with in term of the isolation of chloroplasts.

Again, in order to obtain accurate results, the isolated chloroplasts must be sufficiently pure and intact to adequately study the function in the intact cell. The results of this procedure normally depend on certain main features such as the ratio between the amount of green tissue and the volume of isolation buffer, the type of homogenization equipment employed as well as the size and speed of the polytron rotor, the size of the homogenization beaker, the repetition of the homogenization steps and finally the use of the usual Percoll gradient preparation[7]. In this research, when we established a chloroplast isolation protocol that can be used with the young leaves of *in vitro A. vera* plants, we tried to fit these features into our protocol. The results obtained are shown in fig. 6.

In the above described protocol, the distribution of chloroplasts between the two bands was able to be detected. The upper band contains broken chloroplasts while the lower band contains the intact chloroplasts (Fig. 6a). The integrity of the isolated chloroplasts was confirmed using the transmission electron microscope. The typical micrograph is shown in fig. 6b. From these results, it is possible to suggest that our protocol is suitable for isolating pure and intact chloroplasts from *in vitro A. vera* plants.

It has been indicated that PEPC and COX were localized unambiguously in the cytosol and mitochondria of plant mesophyll cells respectively. In addition, Rubisco is well known as a specific enzyme of chloroplasts that can be isolated from photosynthesis cells so that their activities can be used as the indicators of chloroplast purity. Based on these points, the activities of these enzymes in both leaf extracts and Percoll chloroplasts confirm the contamination of the isolated, intact Percoll chloroplast preparations with other organelles during the process of chloroplast isolation.

In the chloroplasts isolated from *A. vera in vitro* plants, the specific activity of Rubisco was about 138±7 nmolmin^{-1}mg^{1} protein and this value was about 86.25 % of Rubisco activities from the leaves (Table 2). The PEPC was approximately 7 % of that in leaves of *A. vera in vitro* plants (Table 2). The COX activity was about 7.67 % of that value in the leaf extracts on a protein basis (Table 2). These results indicated that the cytosol and mitochondria contamination in Percoll chloroplasts was acceptable and the preparation specifically reflected the chloroplasts properties.

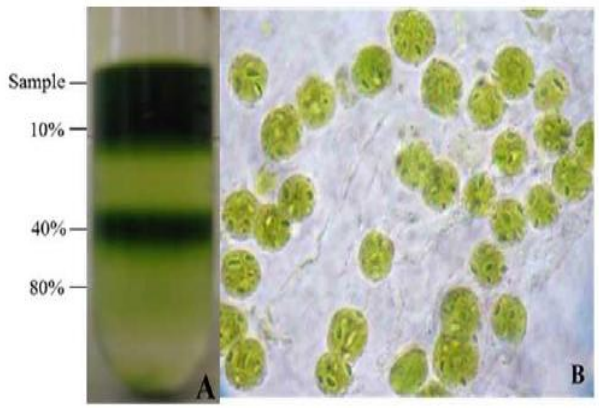

Fig. 6: Isolation of intact chloroplasts from *in vitro Aloe vera* plants, a: Typical two step Percoll gradient showing the distribution of chloroplasts between the two bands and b: Electron micrograph of an intact chloroplast from the lower band of the Percoll gradient.

Conclusion

The high, intact yields of chloroplasts obtained from this research suggest strongly that the protocol implemented may be useful for identifying and characterizing many different aspects of the function and overall role of *A. vera* chloroplast in CAM photosynthesis *in vitro*.

Table 1
Sets forth the contents of water, protein, chlorophyll (Chl) pigments (ratio of the fresh weigh, FW) and photosynthesis intensity of *A. vera* plants after 3 months growth up in the greenhouse.

Water content [% FW]	Protein content [mgg^{-1} FW]	Chl a [μgg^{-1}FW]	Chl b [μgg^{-1}FW]	Chl a / Chl b ratio	Photosynthesis intensity (μmol $CO_2m^{-2}s^{-1}$)
95.2 ± 0.3	0.965 ±0.210	482.5 ± 4.3	279.3 ± 1.4	1.73	7.80 ± 0.24

The mean values ± SD (n = 3) are shown.

Table 2
Enzyme activities in the leaf extract and Percoll-purified *A. vera* chloroplasts

Enzyme	(nmolmin^{-1}mg^{-1} protein)	
	Leaf	**Chloroplast**
Rubisco (EC 4.1.1.39)	160 ± 38	138 ± 7
PEPC (EC 4.1.1.31)	215 ± 11	3 ± 1
Cyt. c oxidase (EC 1.9.3.1)	32.5 ± 1.3	2.3 ±0.2

Results shown are means ± SE (n= 4-5) of separate preparations.

Acknowledgement
This work was supported by a grant of the Vietnam National Foundation for Science and Technology Development (NAFOSTED).

References
1. Abadi D.H. and Kaviani B., *In vitro* proliferation of an important medicinal plant Aloe- a method for rapid production, *Australian J. of Crop Sci.,* **4(4)**, 216-222 **(2010)**

2. Aggarwal D. and Barna K.S., Tissue culture propagation of elite plant of *Aloe vera* Linn, *J. Plant Bioch Biotech,* **13(1),** 77-79 (**2004**)

3. Ahmed S., Kabir A.H., Ahmed M.B., Razvy M.A. and Ganesan S., Development of rapid micropropagation method of *Aloe vera* L., *Sjemenarstvo*, **24(2)**, 121-128 (**2007**)

4. Albanyl N.J., Vilchez S., Lion M.M. and Chacin P., A methodology for the propagation in edge *Aloe vera* L., *Rev. Fac. Agron.,* **23**, 213-222 (**2006**)

5. Anshoo G.S., Singh S., Kulkarni A., Pant S. and Vijayaraghavan R., Protective effect of *Aloe vera* L. gel against sulphur mustard-induced systemic toxicity and skin lesions, *Indian J. of Pharm.,* **6**, 23-29 (**2005**)

6. Arnon D.I., Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta* v*ulgaris*, *Plant Physiol.,* **24,** 1-15 (**1949**)

7. Aronsson H. and Jarvis P., A simple method for isolating import–competent Arabidopsis chloroplasts, *FEBS Letters*, **529**, 215-220 (**2002**)

8. Bradford M.M., A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.,* **72**, 248-254 (**1976**)

9. Campestrini L.H., Kuhnen S.P., Lemos M.M., Bach D.B., Dias D.B. and Maraschin M., Cloning protocol of *Aloe vera* L. as a study-case for "Tailor-Made" biotechnology to small farmers, *J. of Tech Management and Innovation,* **5,** 76-79 (**2006**)

10. Chaturvedi H.C., Jain M. and Kidwai N.R., Cloning of medicinal plants through tissue culture-A review, *Indian J. of Experiment Botany,* **45**, 937-948 (**2007**)

11. Daneshvar M.H., Moallemi N. and Zadeh N.A., The effects of different media on shoot proliferation from the shoot tip of *Aloe vera* L., *Jundishapur J. of Natural Pharm. Products,* **8(2)**, 93-97 (**2013**)

12. Debiasi C., Silva C.G. and Pescador R., Micropropagation of *Aloe vera L, Rev. Bras. Plant Med. Botucatu*, **9**, 36-43 (**2007**)

13. Du Y.C., Nose A., Kawamitsu Y., Murayama S., Wasano K. and Uchida Y., An improved spectrophotometric determination of the activity of ribulose 1,5-bishosphate carboxylase, *Japanese J. Crop Sci.,* **65**, 714-721 (**1996**)

14. Gui Y.L., Xu T.Y., Gu S.R., Liu S.Q., Zhang Z. and Sun G.D., Studies on stem tissue culture and organogenesis of *Aloe vera*, *Acta Botanica Sinica*, **32(8)**, 606-10 (**1990**)

15. Hosseini R. and Parsa M., Micropropagation of *Aloe vera L.* grown in south Iran, *Pak. J. Biol. Sci*., **10(7),** 1134-1137 (**2007**)

16. Keech O., Dizengremel P. and Per Gardeström P., Preparation of leaf mitochondria from *Arabidopsis thaliana*, *Physiol. Plant*, **124**, 403-409 (**2005**)

17. Kondo A., Nose A. and Ueno O., Leaf inner structure and immunogold localization of some key enzymes involved in carbon metabolism in CAM plants, *J. of Exper Botany,* **49**, 1953-1961 (**1998**)

18. Lang E.G.E., Stefanie J.M., Sebastian N.W.H., Joanna P.A., Marco V.S.R and Ralf O. Simultaneous isolation of pure and intact chloroplasts and mitochondria from moss as the basis for sub-cellular proteomics, *Plant Cell Rep*., **30**, 205-215 (**2011**)

19. Liao Z., Chen M., Tan F., Sun X. and Tang K., Micropropagation oendangered Chinese aloe, *Plant Cell Tissue Organ Cult*., **76**, 83-86 (**2004**)

20. Mamidala P. and Nanna R.S., Efficient *in vitro* plant regeneration, flowering and fruiting of dwarf Tomato cv. Micro-Msk, *Plant Omics J*., **2 (3)**, 98-102 (**2009**)

21. Meyer H. and Van Staden J., Rapid *in vitro* propagation of *Aloe barbadensis* Mill, *Plant Cell Tissue Organ Cult*., **26(3)**, 167-71 **(1991)**

22. Møller I.M. and Palmer J.M., Direct evidence for the presence of a rotenone-resistant NADH dehydrogenase on the inner surface of the inner membrane of plant mitochondria, *Physiol. Plant*, **54**, 26-7274 **(1982)**

23. Murashige T. and Skoog F., A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant*, **15(3)**, 473-97 **(1962)**

24. Natali L., Sanchez I.C. and Cavallini A., *In vitro* culture of *Aloe barbadensis* Mill., Micropropagation from vegetative meristems, *Plant Cell Tissue Organ Cult.*, **20(1),** 71-74 (**1990**)

25. Parmar Vilas R. and Jasrai Yogesh T., Micropropagation of an Important Medicinal Plant Aloe barbadensis Mill (Aloe vera L) for Field Plantation, *Res. J. Biotech*., **4(1)**, 7-10 **(2009)**

26. Sanchez I.C., Natali L. and Cavallini A., *In vitro* culture of *Aloe barbadensis* Mill, Morphogenetic ability and nuclear DNA content, *Plant Sci.,* **55**, 53-59 **(1988)**

27. Shaheen A., Nose A. and Wasano K., *In vivo* properties of phospho*enol*pyruvate carboxylase in crassualcean acid metabolism plant-Is pineapple CAM not regulated by PEPC phosphrylation?, *Environment Control in Biology,* **40**, 343-354 **(2002)**

28. Stern D.B., Hanson M.R. and Barkan A., Genetics and genomics of chloroplast biogenesis, maize as a model system, *Trends in Plant Science*, **9(6)**, 293-301 (**2004**)

29. Truong T.B.P., Tung N.T., Thanh T.T. and Hien N.T., *In vitro* propagation of a medicinal plant-*Aloe vera* L., *Vietnamese J. Biotechnol.,* **8(3B)**, 1221-1229 (**2010**)

30. Velcheva M., Faltin Z., Vardi A., Eshdat Y. and Perl A., Regeneration of *Aloe arborescens* via somatic organogenesis from young inflorescences, *Plant Cell Tissue Organ Cult.,* **83(3)**, 293-301 **(2005)**

31. Zhi-fang L., Gui-zhu L., Gu-chou S. and Shuang-shun L., The relation between activity of phosphoenolpyruvate carboxykinase and photosynthesis in *Aloe vera* leaves, *Acta Botanica Sinica*, **33 (4),** 273-279 **(1991).**

(Received 14th July 2013, accepted 20th September 2013)