World Academy of Science, Engineering and Technology
International Journal of Biomedical and Biological Engineering
Vol:8, No:12, 2014

Indirect Regeneration and Somatic Embryogenesis from Leaf and Stem Explants of *Crassula ovata* (Mill.) Druce – An Ornamental Medicinal Plant

A. B. A. Ahmed, Amar, D. I., R. M. Taha

***Abstract*—**This research aims to investigate callus induction, somatic embryogenesis and indirect plant regeneration of *Crassula ovata* (Mill.) Druce – the famous ornamental plant. Experiment no.1: Callus induction was obtained from leaf and stem explants on Murashige and Skoog (MS) medium supplemented with various plant growth regulators (PGRs). Effects of different PGRs, plant regeneration and subsequent plantlet conversion were also assessed. Indirect plant regeneration was achieved from the callus of stem explants by the addition of 1.5 mg/L Kinetin (KN) alone. Best shoot induction was achieved (6.5 shoots/per explant) after 60 days. For successful rooting, regenerated plantlets were sub-cultured on the same MS media supplemented with 1.5 mg/L KN alone. The rooted plantlets were acclimatized and the survival rate was 90%. Experiment no.2: Results revealed that 0.5 mg/L 2,4-D alone and in combination with 1.0 mg/L 6-Benzyladenine (BA) gave 89.8% callus from the stem explants as compared to leaf explants. Callus proliferation and somatic embryo formation were also evaluated by 'Double Staining Method' and different stages of somatic embryogenesis were revealed by scanning electron microscope. Full Strength MS medium produced the highest number (49.6%) of cotyledonary stage somatic embryos (SEs). Mature cotyledonary stage SEs developed into plantlets after 12 weeks of culture. Well-rooted plantlets were successfully acclimatized at the survival rate of 85%. Indirectly regenerated plants did not show any detectable variation in morphological and growth characteristics when compared with the donor plant.

***Keywords*—**Callus induction, *Crassula ovata*, Double Staining, Indirect plant regeneration, Somatic embryogenesis.

I. Introduction

CRASSULA OVATA (Mill.) Druce, a member of succulence plants is a vascular and slow growing plant of family Crassulaceae. Local Malaysian refers to this plant as 'jade plant' or 'money tree'. It is a popular houseplant and perhaps the most common container plant. Plant tissue culture offers a viable alternative for propagation via somatic embryogenesis and plant regeneration. The demand for ornamental and medicinal plants is therefore very high leading to their over-exploitation from the wild population. Therefore, an efficient propagation protocol needs to be developed for this species for its application in traditional medicine and commercial planting.

A.B.A. Ahmed is with the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. (Corresponding author to provide phone: +6037967-6768; fax: +60379674178; e-mail: bakrudeen@um.edu.my).

D.I. Amar and R.M. Taha are with the Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia.

A. Medicinal Plants

Medicine in several developing countries, using local traditions and beliefs, is still the mainstay of health care. As defined by WHO, health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity [1]. Medicinal plants, since time immemorial, have been used in virtually all cultures as a means of remedy and cure. Developed countries, in recent times, are turning more and more to the use of traditional medicinal systems that involve the use of herbal drugs and remedies. According to a recent survey in member states of European Union about 1400 herbal preparations are used widely [2]. An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extractions and development of several drugs and chemotherapeutics from these plants as well as from several traditionally used herbal remedies. Despite the increasing use of medicinal plants, their future is being threatened by complacency concerning their conservation. Reserves of herbs and stocks of medicinal plants in developing countries are diminishing and are in danger of extinction as a result of growing trade demands for cheaper healthcare products and new plant-based therapeutic markets in preference to more expensive target specific drugs and biopharmaceuticals. Such concerns have stimulated positive legal and economic interest. Use of aromatic herbs and species by the antiquity is well documented in ancient writings, Old Testament, early Greek and Roman documents, in medieval manuscripts. Species mainly depend upon the chemical constituents of essential oil for their aroma and flavoring characteristics. With the development of botanical drugs, including traditional herbal medicines, analysis of their bioactive components is becoming more popular. Medicinal plants make an important contribution to the WHO goal to ensure by the year 2000 that all people worldwide will lead a sustainable socioeconomic life. Genetic biodiversity of traditional medicinal herbs and plants is continuously under the threat of extinction as a result of growth exploitation, environmentally unfriendly harvesting techniques, loss of growth habitats, and unmonitored trade of medicinal plants. With a growing demand for the economic products of plant origin, relentless efforts are being made to enhance plant productivity and quality of produce, and, to develop plants of agronomic value through newer technologies including biotechnology.

B. Plant Growth Regulators

Plant contains chemical substances that can be altered the plant growth and organ development processes are quite natural; in terms of macronutrients, micronutrients, vitamins, iron sources and PGRs respectively. Therefore, PGRs generally include some compound that promotes or inhibits plant growth and development. These compounds may be synthetic chemical or the natural products of plant cells. PGRs may indirectly control gene expression through biosynthetic pathway enzymes and messengers by acting during transcription, mRNA processing, mRNA stability, and at translational and post-translational modifications. PGRs are corresponding to the signaling cascades, namely, the signal generator, their receptors, messengers and intracellular targets, as well as the pathways of signal transduction to the targets and back remains a primary take of molecular physiology. Communication by extracellular signals usually involve different stages *viz.,* synthesis; release of the signaling molecule by the signaling cell; signal transmission to the target cell; detection of the signal by a specific receptor protein; a change in cellular metabolism, function, or development that is triggered by the receptor-signal complex; and finally, the termination of cellular responses. PGRs, used as trace signal molecule in plants, has very important significance in regulating all kinds of growth processes and environmental responses, and meanwhile, made great contribution to the agricultural chemical control of crops, fruits and vegetables.

C. Auxins

Auxins influence virtually every aspect of plant growth and development, numerous bioassay for auxin response have been described. As critical plant hormone, auxin has modulates such diverse processes as tropic responses to light and gravity, general root and shoot architecture, organ patterning, vascular development and growth in tissue culture. *In vitro* induced auxins seem to be capable of erasing the genetically programmed physiology of whole plant tissue, which had previously determined their differentiated state. The effect of auxins depends on the magnitude of dedifferentiating effect of the auxins applied. Strong auxins have specific inhibitory effect in which, secondary product accumulate in tandem with a special mechanism via direct influence on enzymatic activities and via the influence on transcription. Auxins can also promote plant sex differentiation, the formation of flowers and to promote the formation of seedless fruit. Auxins main use in tissue culture to induce callus and, also they are important use is in line with a certain amount of cytokinin for root differentiation, lateral bud germination and growth, as well as in certain plants induce embryogenesis. Since, many research articles have dealt at great length about the status, applications, potentials and needs in tissue culture of ornamental plants. Since, the major effects have been made to develop basic back ground technologies for consistent production and regeneration of calluses from diverse group of medicinal plants. The techniques which have been so far described for propagation of ornamental plants through tissue culture have been tested on the laboratory scale and have not been validated for their suitability in commercial scale production. However, the following aspects have to be critically studied if the economic prospects associated with *in vitro* culture technology are to be realized. Generally, the application of plant cell and tissue culture technique which is commonly known as *in vitro* cloning can also be divided into several procedures, including meristematic cultures, vegetative explant cultures, callus induction, suspension cultures, direct and indirect somatic embryogenesis, synthetic seed production, *in vitro* flowering, *in vitro* mutation breeding, protoplast and also somatic hybridization process. Some of these techniques would selectively be applied to selected plants overcome generation incapability of the plants. This *in vitro* technique is very useful in ensuring sustainable, optimized sources of plant-derived natural products. However, ex situ cultivation should be preceded by proper evaluation of the plants for their ability to produce the required bioactive constituents before commencing cultivation or introducing the technology to potential growers. The ability of plants to produce certain bioactive substances is largely influenced by physical and chemical environments in which they grow. Plants also produce certain chemicals to overcome abiotic stresses. In this aspects plant tissue culture developed callus influenced by medium, explants, plant growth regulators, color lights, temperature, photoperiod and carbon sources are helpful to produce valuable secondary metabolites compounds in many studies. Growing a plant outside its natural environment under ideal conditions may therefore, result in being unable to produce the desired bioactive substances, hence the need for prior evaluation.

D. Cytokinins

Cytokinins appear to be necessary for plant cell division. It was observed that cytokinin absence, lead to the considerable protraction in metaphase, but not the prophase of mitosis, which suggested that cytokinins might be required for the regulation and synthesis of proteins involved in the formation and function of the mitotic spindle apparatus. When cultures, the cytokinin is limiting, division of cell nuclei becomes arrested at one stage of the cell cycle. Subculture of the tissue onto medium containing cytokinins can then cause the cells to divide synchronously of the lag period. Cytokinins are purine derivatives with phytohormone activity that can influence several plant processes such as growth of lateral buds, leaf expansion and leaf senescence. They can also influence (in combination with auxins) cell division and morphogenesis in *in vitro* plant tissue culture. Since, the scientists are considered cytokinin is catalyzed by cytokinins dehydrogenase, a flavoprotein oxidoreductase, which reacts poorly with oxygen and prefers quinine-type electron acceptors.

E. Plant Propagation Applications

The plant is used extensively for conservation, propagation and bio-active compounds production. The systematic manipulation of media, phyto-hormone concentrations and

selection of suitable explants are amongest several key factors that control the process of somatic embryogenesis from callus; the callus-mediated regeneration (indirect regeneration) protocol is a crucial requirement for the horticulture and pharmaceutical industry. The principal advantage of this technology in pharmaceuticals is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. In addition to its importance in the discovery of new medicines, plant tissue culture technology plays an even more significant role in solving world hunger by developing agricultural crops that provide both higher yield and are more resistant to pathogens and adverse environmental and climatic conditions. Plant regeneration can be achieved in two ways: through organogenesis and somatic embryogenesis. This study aims to establish efficient callus induction and high frequency of plant indirect regeneration and somatic embryogenesis [3]. To the best of our knowledge, no study regarding organogenesis from callus and somatic embryogenesis from leaf and stem explants has been reported in *Crassula ovata*.

II. Materials and Methods

A. Plant Collection

Crassula ovata was purchased from Carrefour Mid-valley Megamall, Kuala Lumpur, Malaysia. It was maintained at the ISB garden for further studies (Figs. 1 (a) and (b)). Leaf and stem were used as explant source.

B. Surface Sterilization

Both explants were pre-rinsed under running tap water for 30 min, followed by 10% of sodium hypochlorite (chlorox) and 2 to 3 drops of tween 20 was used to wash the explants for 1-2 min. Explants were rinsed using Sterile Distilled Water (SDW) to remove any possible residues and was repeated 5 times.

C. Callus Induction

The surface sterilized explants were inoculated on MS media [4] and various concentrations of PGRs ranging from (0.5mg/L-2.0mg/L) of 2,4-Dichlorophenoxyacetic acid (2,4-D), Napthaleneacetic acid (NAA), Kinetin (KN), 6-Benzyladenine (BA) and Indole Acetic acid (IAA), either alone or in combinations were added. Sucrose (3%) was added as the source of carbon (Himedia®, Malaysia) and agar technical powder was used to solidify the media.

D. Culture Conditions

The pH of the media was maintained at 5.8 and autoclaved at 121˚C with a pressure of104 K Pa for 20 min. Cultures were kept at temp of 25±2˚C for 16 – 18h (dark/light) under a 25 µ mol m^{-2} s^{-1} of light intensity period and with relative humidity of 65-75%. The callus initiation, fresh weight, dry weight and nature were determined in all callus culture at weekly interval before subculture.

E. Statistical Analysis

The somatic embryogenesis and plant regeneration experiments followed a completely randomized design. All experiments were repeated thrice. Thirty replicate cultures were rinsed for each treatment. All the observed data was subjected to statistical analysis using Duncan's multiple range test (DMRT) at 5% level of significance [5].

Fig. 1 (a) and (b) *Crussula ovata* mother plant

III. Results and Discussion

In MS medium without PGRs, the callus initiation failed in stem and leaf explants of *C. ovata* (data not shown). Ahmed, Rao et al. reported that the factors such as type of explants, PGRs, culture media and cultural conditions play an important role in plant propagation [3]. The suitable indirect regeneration callus induction was observed in MS media containing 2,4-D. All concentration ranges from 0.5-2.0 mg/L 2,4-D gave friable, slightly red, yellow and pale-green callus formation from stem and leaf explants which remarkably turned out to be the highest (100%) maximum with a mean dry weight.

A. Organogenesis

Both the stem and leaf explants showed successful regeneration depending upon different concentrations of PGRs. In the case of *Crassula ovata*, formation of shoots from stem explants showed best results, yielding 100% as compared to leaf explants. Shoot regeneration responded well at concentrations of 1.5 mg/L KN with 100% shoots formation. In addition, BA and KN combinations were tried for callus regeneration, the results showed less number of shoots and shoot length stunted than 2,4-D and NAA combine with BA and KN concentrations. In *Phyla nodiflora* callus regeneration protocol suggested that BA and KN combinations showed less number of shoots and stunted shoot length than auxins and cytokinins concentrations [6].

B. Somatic Embryogenesis

Embryogenic tissue was maintained and bulked up through secondary somatic embryogenesis. In order to stimulate SE maturation, the pieces of embryogenic tissue (15 to 20 mg), which considered of immature embryos, were transferred into each conical flask containing suspension maturation medium supplemented with plant growth regulators. The highest frequency embryos at the globular stage, heart stage were observed onto MS media supplemented with auxins and cytokinins treatments. During the embryos maturation, the batch culture was continuously subculture every week to prevent the re-callus and phenolic excretion in the medium.

C. Acclimatization

The MS basal medium was added with 30g/L sucrose, at $_{P}$H 5.7±0.1 and solidified with 2g/L gelrite. The cultures were maintained in the culture room at 25±2˚C under16 hours of light and 8 hours of dark period. Significantly reduced abnormal embryos were observed in MS medium (data not shown). The plantlets were transferred to black soil in vases covered with plastic bags and acclimatized in the culture room for 2 months (Figs. 2 (a) and (b)). Subsequently, the plantlets were transferred to a greenhouse and their ability to fully adapt to the natural environment was monitored.

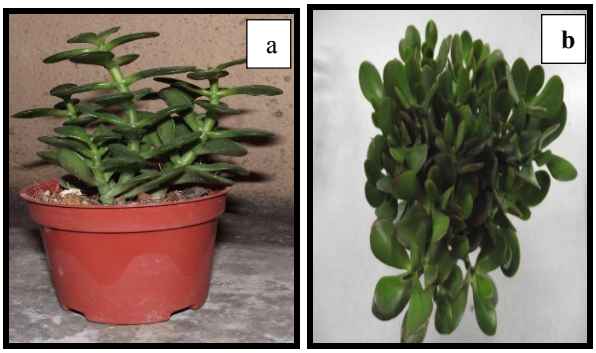

Fig. 2 (a) and (b) Acclimatization plant of *Crussula ovata*

IV. Conclusion

The growing demand for ornamental and medicinal plant cultivation of this necessitates an alternative faster rate of multiplication. This plant regeneration protocol could be used to produce uniform quality disease free plant at a faster rate with in a limited space. The efficient somatic embryos protocol could be useful for conservation and agronomy and in the improvement of *Crassula ovata* using gene transfer biotechnologies.

V. Acknowledgement

The authors would like to thank University of Malaya for the financial support (RP025-2012B) and facilities provided to successfully carry out research.

References

[1] WHO/Acadia, Rapport de la Journe Internationale de, diabetes, 1992, 14 Octobre.
[2] H. Lucy, and J. D. Edgar, "Medicinal Plants: a re-emerging Health aid" Plant Biotechnology, 2, 1999, 3-8.
[3] A. B. A. Ahmed, A. S. Rao, M. V. Rao, and R. M. Taha, "Effect of picloram, additives and plant growth regulators on somatic embryogenesis of *Phyla nodiflora*", Brazilian Archives Biology and Technology, 54, 2011, 7-13.
[4] T. Murashige, and F. A. Skoog, "Revised media for rapid growth and bioassay with tobacco tissue cultures", Physiology plant, 15, 1962, 473-497.
[5] K. A. Gomez, and A. A. Gomez, "Statistical procedures for agricultural research with emphasis on rice", International Rice Research Institute, Los Bans, Philippines, IRRI Publication, 1976, p. 264.
[6] A. B. A. Ahmed, A. S. Rao, M. V. Rao, and R. M. Taha, "Optimized conditions for callus induction, plant regeneration and alkaloids accumulation in stem and shoot tip explants of *Phyla nodiflora*", Spanish Journal of Agricultural Research, 9, 2011, 1262-1270.