Bangladesh J. Bot. **50**(2): 301-309, 2021 (June)

PRODUCTION OF LIMONOIDS THROUGH CALLUS AND CELL SUSPENSION CULTURES OF CHINABERRY (*MELIA AZEDARACH* L.)

NADIR ALI RIND[1*], MUHAMMAD RAFIQ[1], MUHAMMAD UMAR DAHOT[1], HAFIZA FAIZA[1], ÖZLEM AKSOY[2], KHALID HUSSAIN RIND, AKHTAR HUSSAIN SHAR, HIDAYAT ULLAH AND ASHIQUE HUSSAIN JATOI[3]

Department of Molecular Biology and Genetics, Shaheed Benazir Bhutto University, Shaheed Benazir Abad, Pakistan

Keywords: Chinaberry, Cell suspension culture, Limonoids

Abstract

To study the *in vitro* production of limonoid contents through callus and cell suspension cultures of chinaberry (*Melia azedarach* L.) in different explants were inoculated in Murashige and Skoog (MS) medium for the callus induction with various plant growth regulators (PGRs) separately as well as in combinations. The highest callus induction (73.3%) was observed in nodular stem sections and further callus was subcultured for multiplication and finally transferred to cell suspension medium. The optimized parameters for the production of total limonoids were adjusted and UV-visible spectrophotometer was used to quantify total limonoids at 577 nm. Production of total limonoids through callus cell suspension cultures on the optimized medium was highest (141.7 µg/ml) followed by other sources like KH_2PO_4 (0.1 g/l) extra supplementation with optimized medium produced (138 µg/ml). Plant callus cell suspension cultures through optimized medium may be considered as a good source for the production of bio-products and its purified form could be used as a medicinal sources.

Introduction

The Chinaberry (*Melia azedarach* L.) belonging to Meliaceae is widely distributed throughout the tropics, subtropics and temperate zones. It is a fleshy-fruited small to medium-size tree native to northwestern India. Chinaberry plant has been introduced to many subtropical countries is also cultivated in different areas of Pakistan up to 1700-meter height which can be used as ornamental shade-tree and also as insect repellent (Hammad *et al*. 2000 and Nasir *et al.* 1972). In last 20 years, this family identified as one of the most important sources of bioproducts of genera *Melia* and *Azadirachta*, were found very effective (Schmutterer and Wilps1995).

Plants growing in natural environment defend themselves against biotic constraints to maintain their survival, and that they synthesize different secondary metabolites having important physiological and ecological functions was reported by Guerriero *et al*. (2018), Jassim and Naji (2003) and Wallace (2004). *M. azedarach* is a multipurpose tree because it is used in multidirectional and widespread purposes in medicine, therapeutics as well as for other economic implications (Shekhawat *et al*. 2014). Secondary metabolites are the main constituents of plants involved in the protection against herbivores, bacteria, viruses, fungi and other competing with plants. Some plants made use of bio-products as serve to attract pollinators and seed dispersers (Wink 2003, Yang *et al*. 2018). Plant secondary metabolites are biologically active, non-nutrients and having good role in the maintenance of human life are used as food, drink (Cherif 2012). A large number of these plant-based chemicals are used as important sources for varieties of pharmaceuticals, agrochemicals, food additives, flavors, pesticides, resins, dyes and oils (Balandrin and Klocke1988, Parr 1988).

*Author for correspondence: <nadirali.rind@sbbusba.edu.pk>, [1]Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan. [2]Department of Biology, Faculty of Arts and Science, Kocaeli University, Turkey, [3]Department of Chemistry, Shaheed Benazir Bhutto University, Shaheed Benazir Abad, Sindh, Pakistan.

DOI: https://doi.org/10.3329/bjb.v50i2.54086

Chinaberry is rich source of secondary compounds including tetranortriterpenoids (limonoids) which are important phytochemicals due to their wide variety of properties like antioxidant, antimalarial, antibacterial, antifungal and insecticidal effects against disease-causing vectors (Coria *et al*. 2008, Khanavi *et al*. 2008, Mazumder and Rahman 2008, Thirumalai *et al*. 2009, Abdel-Aty and Abdel-Aty 2015). In some countries, the chinaberry plant is used for the medicinal purpose of treating humans against different diseases such as nausea, vomiting, skin diseases, anthelmintic, diuretic, vermifuge, typhoid fever, and pain in the pelvic region and also for the treatment of asthma (Hammad *et al*. 2001). Fruit extracts of chinaberry have multiple effects against insects like growth retardation, antifeedant, moulting disorders and changes of behavior (Schmidt *et al*. 1998, Hammad *et al*. 2001, Wandscheer *et al*. 2004).

The amount of plant based medicinal and insecticidal compounds in different parts of chinaberry are low. This might be due to effect of geographical distribution of plants as well as seasonal variations affecting the production of these limonoids from targeted plants. Similarly, that these plants may be susceptible to environmental influences like light, heat, etc. was reported by Sundaram *et al*. (1995), Szeto and Wan (1996) and Jarvis (1998). The plant products (secondary metabolites) are limited due to above mentioned problems for production. As awareness towards safer, environment friendly plant products are increasing, demand of chinaberry based limonoids is also increasing continuously. Limonoids have complex chemical structures, which are difficult to ways in the laboratory. To overcome these problems, there is urgent need to develop alternative route for limonoids production. The use of biotechnological techniques like plant cell and tissue culture for production of these metabolites is getting importance. That *in vitro* callus is an alternative source for the production of secondary metabolites was reported by Samar *et al*. (2013). The advantage of *in vitro* technique is the continuous production of these metabolites, which is not affected by any seasonal variation or other environmental influences and diseases. Thus the plant cell and tissue culture technique can be used for the enhanced production of these metabolites (Ushiyama and Hibino 1997, Ferri *et al*. 2011).

Callus cell suspension culture is an efficient means for the production of secondary metabolites with the addition of various plant growth regulators (Zeng *et al*. 2009). There are also a few reports on the production of azadirachtin related limonoids (Rafiq 2010) and azadirachtin (Prakash *et al*. 2002, Sujanya *et al*. 2008) through callus and cell suspension cultures of neem. The work on the production of these bioactive compounds through callus cell and tissue culture is very important, supporting that *M. azedarach* cell and tissue cultures can be established and may be used as an alternative source for the production of limonoids. The limonoid compounds may be produced in any aseptic place/conditions without any changing season and in at low cost. Attempts were made to develop desired medium with the addition of different concentrations of sucrose.

Materials and Methods

Different explants (Immature flowers, nodular stems, immature embryos, leaves and petioles) of chinaberry plants were collected from Sindh University, Jamshoro, Pakistan. The whole experimental work was carried out at Plant Tissue Culture Lab., Institute of Biotechnology and Genetic Engineering, University of Sindh, Jamshoro, Pakistan. Experiment was carried out following the method reported by Rafiq and Duhot (2010). Initially samples were washed with distilled water thoroughly sterilized with specified concentration of sodium hypochlorite (commercial bleach) for 12 - 17 min, incubated in methanol for one minute, finally washed three times in sterile distilled water. Sterilization was carried out in laminar airflow cabinet.

Various explants cut in to small pieces (about 2 mm were in MS semi-solid medium with 1.0 mg/l 2,4-D, 5.0 mg/l GA_3 and 3.0 mg/l NAA. Then cultures were left at 26 ± 2°C and 10/14

dark/light photoperiod for the callus induction. Cultures were maintained under these conditions and then transferred to the suspension liquid media after homogenization in optimized conditions.

Friable callus 0.5 g of fresh callus was taken from semi-solid MS medium, crushed and converted into small pieces and transferred to 5.0 ml MS liquid medium containing 1.0 mg/l of each 2,4-D, 5.0 mg/l GA_3 and 3.0 mg/l of NAA for homogenization. Then suspended homogenized callus cells in liquid media were transferred picking 2.5 ml in 30 ml suspension medium then left on shaking incubator at speed 200 rpm at 26 ± 2°C 18 days of incubation. Media and cultures broth were separated, and methanol was used as a solvent for the extraction of limonoids from cultures broth as well as from biomass.

Different parts of *M. azedarach* were dried at 50°C for 48 hrs, and then 3 g were ground in pestle mortar with autoclaved distilled water and 90% methanol separately. Extracts were centrifuged at 7000 rpm for 10 min, then the supernatant was taken and adjusted the volume up to 30 ml and finally samples were stored at – 40°C. The dry callus ground 0.5 g in pestle mortar using autoclaved by D H_2O and 90% methanol separately. Sample was centrifuged at 7000 rmp for 10 minutes then separated the supernatant finally adjusted volume up to 5 ml with distilled water and methanol separately. The extraction process was repeated twice. Samples were stored at –40°C for further chemical analysis.

Total limonoid contents were quantified following the method reported by Dai *et al.* (1999), 0.7 ml test sample plus 0.2 ml of methanol solution of vanillin (0.02 mg/ml) were mixed then samples were shacken manually and left at room temperature for 2 min. The 0.3 ml concentrated H_2SO_4 was added in three portions (0.1 ml each), and the mixture was stirred for 10 sec after each addition. With the addition of 0.7 ml methanol, the solution converted into two-layer mixture of a blue-green color. The solution was left at room temperature for 5 min, and then absorbance was noted at 577 nm using UV-visible spectrophotometer.

Per cent callusing response was determined using the method reported of Ding *et al*. (1981). The data were statically calculated by one-way ANOVA test using SAS 16.0 (SAS Inst. Inc., Cary, N.C., and the U.S.A) and Microsoft Excel 2010. The reported results are the average of three replicates and expressed as mean ± Sd.

Results and Discussion

Callus induction was made from in different parts of chinaberry with the addition of different PGRs in the medium as shown in Table 1. Variable callus induction effects were observed with the combination of different PGRs like 2, 4-D, BAP, GA_3 and NAA on different explants of *M. azedarach* (flowers, leaves, embryos, stem and petioles). It is seen (Table 1) that the nodules stem section under dark and light condition produced (73.3%) callus in medium with 1.0 mg/l 2,4-D, 5.0 mg/l GA_3 and 3.0 mg/l NAA as compared to other explants and PGRs combinations. Followed by immature flowers produced 66.6% callus induction response with the supplementation of 1mg/l 2,4-D, 1 mg/l BAP and 0.3 mg/l NAA on the 8th week of incubation (Fig. 1).

Total azadirachtin related limonoid contents determined from direct plant source with 90% methanol was used as a solvent for leaf extracts. It was noted that from direct sources highest total limonoid contents were 80.17 ± 8.9 µg/ml determined from leaf followed by 67.01 ± 4.9 µg/ml from flowers. With the use of water as a solvent 45.80 ± 2.5 µg/ml total limonoid contents were noted (Fig. 2). Limonoid contents from dry callus 0.5 g in 5 ml solvent were determined in immature flowers (40.85 ± 5.3 µg/ml) followed by nodular stem sections (26.24 ± 2.3 µg/ml) in methanol extracts. It was observed that limonoid contents were highest in methanol extracts as compared to water extracts (Fig. 3).

Table 1. Effect of different concentrations of plant growth regulators on callus induction.

Plant growth regulators (mg/ml)					% callusing response of Chinaberry explants				
Group	2,4-D	BAP	GA_3	NAA	Flowers	Leaves	Embryos	Stem sections	Petioles
A	0.5	-	-	-	25.5	21.7	11	56*	3.03
B	1.0	-	-	-	66.2**	16.6	10	46.6	11.7
C	1.0	1.0	-	0.3	33.8	NR	25	NR	11.24
D	1.0	1.5	-	-	NR	20	23.1	50	NR
E	1.0	-	5.0	3.0	50	37.5	12.4	73.3***	12.05
F	1.0	1.0	-	0.3	30.8	NR	22	30.4	NR
G	2.0	-	-	-	37.5	16.6	16	45.4	2.6
H	2.0	1.0	-	1.0	33	NR	NR	40	NR
I	2.0	1.0	-	1.0	NR	33.2	NR	42	NR

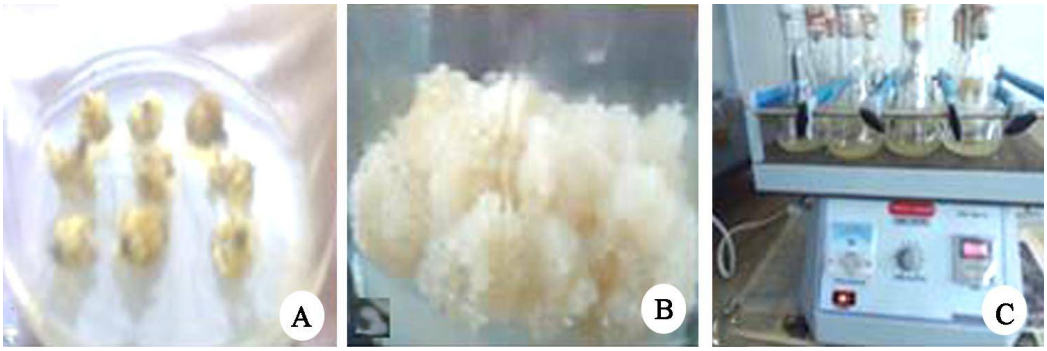
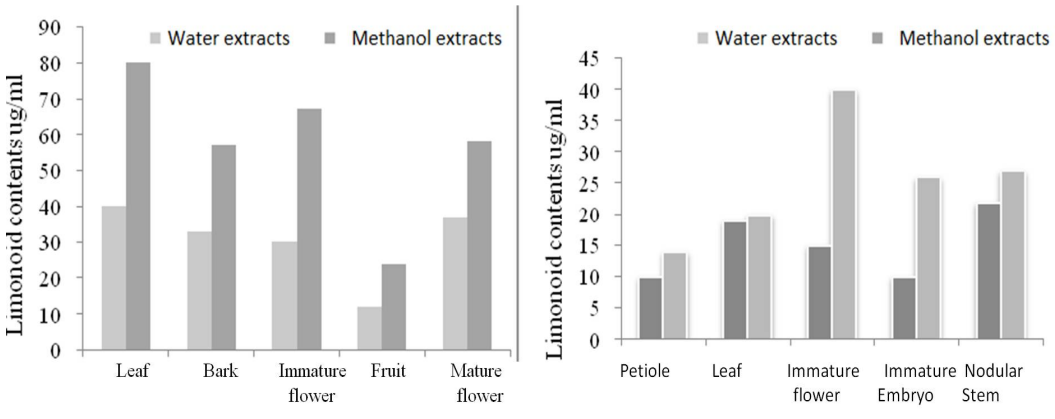


Fig. 1 (A-C) Inoculated flowers, callus and suspension cultures for the production of secondary metabolites.



A: Limonoid contents from intact plant of Chinaberry

B: Limonoid contents from dry callus of different explants

Fig. 2 (A-B). Total limonoid contents from different intact parts of chinaberry and dry callus.

Callus cell suspension culture showed continuous and stable multiplication of biomass. The time taken for cell growth and limonoids production have been studied by growing callus cells in 250 ml conical flask containing MS liquid medium. As shown in Fig. 3, limonoids production was slow during 1-6 days of incubation and its biomass reached the highest value on 18 days of incubation. Sucrose in cell suspension culture hydrolyzed immediately into glucose and fructose after initiation of culture and disappeared in 48 hrs from callus initiation. In the present work, different percentage of sucrose concentrations supplemented to cells suspension culture of *M. azedarach* are shown in Fig. 3. The highest level of limonoids was noted at 03% sucrose 10/14 dark/light photoperiod followed by 4%. The pH of agar medium and suspension culture was adjusted to 5.8 for the proliferation of callus and production of total limonoids. It was most important to investigate the effective pH value of cell suspension culture because production increased or decreased at pH 5.8 as kept for control. Lowest production of limonoids was determined at pH 5.4 and highest at pH 5.8 (Fig. 3) both in biomass and extracellular media (broth). Temperature also effects on the cells in cultures media. Therefore, its optimization is important. Almost cells belong to plant cultures between the range of 20 and 28°C and production of limonoids increased at temperature ranges (24 - 29°C) in suspension culture. The results mentioned in Fig. 3 showed that highest amount of total limonoids was produced at 28°C. As the temperature increased the reduction effect in limonoids was noted.

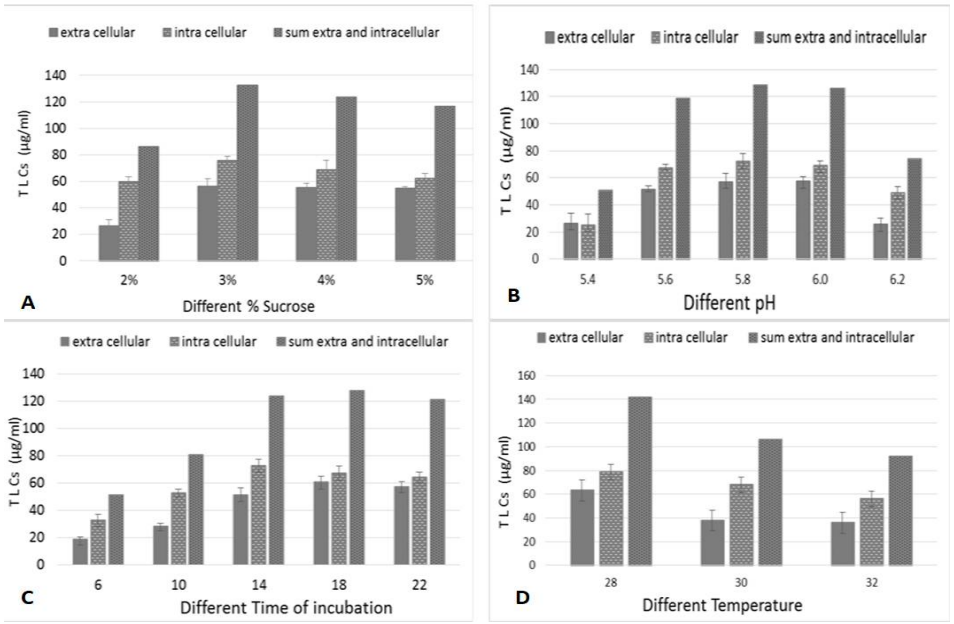


Fig. 3A-D: A: Different % sucrose, B: Different pH, C: Different time of incubation, D: Different temperature.

The phosphate, sulfate and nitrogen sources like KH_2PO_4, NH_4NO_3, KNO_3, NH_4SO_4 and urea $(CO(NH_2)_2$ at different concentration were added in the callus cell suspension culture media for the of production total amount of limonoid. With the addition of all chemicals, overall production of limonoids almost remains the same, although decreased but not increased as compared to without addition of chemicals (Fig. 4) in the presence of optimized chemicals in MS cell suspension culture medium.

The quality and frequency of callus and cell suspension culture depend on optimization of PGRs and culture environment. Like MS medium 2,4-D (1 mg/l), NAA (3.0 mg/l), GA3 (5.0 mg/l) and 3% sucrose were added in MS medium and inoculated at 26 ± 2°C. In present work, supplement of additional chemicals did not increase the product as compared to without chemicals. Good quality of callus either increased yield or improved the nutritional quality.

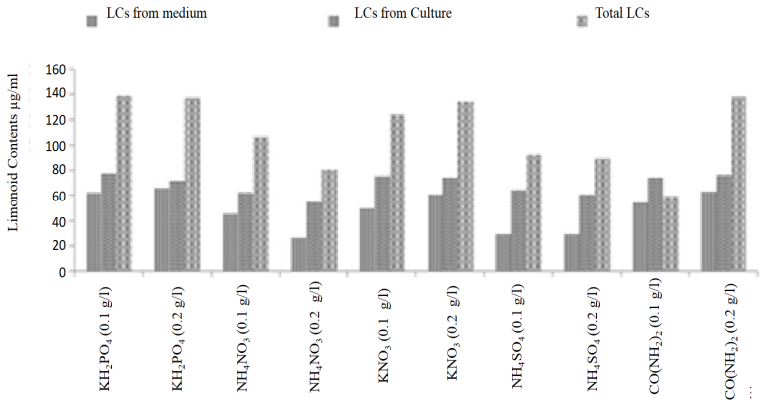


Fig. 4. Showing the effect of different phosphate, sulphate and nitrogen sources on the production of limonoid contents through callus cell suspension cultures.

Plant bio-products produced through cell and tissue culture technology has been extended by Simões *et al*. (2012) for commercial use and tissue culture with optimized conditions like time of incubation, pH, temperature. Different additives like PGRs and nitrogen sources may increase the production through callus cell suspension cultures (Abbas *et al.* 2018). As mentioned in Fig. 3, production of limonoids and biomass were found to be slow and same type of results were reported by Cheng *et al.* (2006) where decreased of biomass and limonoids production after 22 days of time of incubation was observed. After every 18 days of incubation 10% media with suspension culture was transferred to new liquid media having same PGRs for long time stable production of limonoids. Sucrose is an important as carbon and energy source for all plants (Al-Khayri *et al*. 1996, Nhut *et al*. 2000). It was reported that the number of parameters related to secondary metabolites in plant cell cultures such as growth rate and yield are hardly affected by initial sucrose concentrations (Benavides 1997, Wang *et al*. 1997). Sucrose in cell suspension culture hydrolyzed immediately into glucose and fructose after initiation of culture and disappeared in 48 hrs from callus initiation. The present results are closely supported by Babu *et al*. (2008), who worked on enhancement of salannin (limonoid having insecticidal activity) productions in cell suspension cultures of *Azadirachta indica*. Results indicated that changing in sucrose concentration is an effective method for the production of high level of secondary metabolites in cell cultures (Pasqua *et al*. 2005). Optimized pH is extremely important as uptake of nutrients and PGRs are influenced by pH by regulating the solubility in cell suspension medium (Bhatia and Aswath 2005). Most of the reports showed that the pH of plant cell suspension culture media was maintained at 5.8 (Fig. 3), which justifies present results; it may be possible that the stability of azadirachtin is higher in acidic pH (Jarvis *et al*. 1998). It has been reported that *A. indica* cells with the reduction of pH in long term lose viability and died (Potters *et al.* 2007). Temperature also effects on the cell cultures and optimization is important. In present work, the highest amount of total limonoids were produced at 28°C with Choi *et al.* (2000), who reported

that cells belong to plant cultured between was in conformity 20 and 28°C and production of limonoids increased at 24 to 29°C by suspension culture in *Taxux chinensis*.

Optimized production of valuable bioactive compounds through callus cell suspension cultures could be useful for humans and used safely as medicines without any side effects as compared to synthetic medicines. This technique can also be applied to the other medicinal plants for multipurpose applications and may be baseline investigations of the production of limonoids through callus cell suspension under controlled conditions. Most of the medicinal plants could be selected and used for the production of secondary metabolites through callus cell suspension cultures.

The production of limonoids from direct source of *M. azedarach* L. is laborious and much expensive than callus cell suspension culture considered to be the most appropriate method for the production of total limonoids. Optimized conditions as discussed here can be used for the production of biologically active compounds from other medicinal plants. This method could be more valuable alternative for the production of bioactive compounds, which could be used for biological control as well as other medicinal purposes.

Acknowledgements

This work was supported by the Higher Education Commission (HEC) of Pakistan and TUBITAK, Turkey. The author is very much thankful to Prof. Dr. Fazil ÖZEN, Head, Department of Biology, Faculty of Arts and Sciences, Kocaeli University, Turkey for the support to complete the Research project and Prof. Dr. Syed Habib Ahmed Naqvi, Director Institute of Biotechnology and Genetic Engineering (IBGE), University of Sindh, Jamshoro, Pakistan for the facilitation at Institution, generous support and encouragement.

References

Abbas MS, El-Shabrawi HM, Soliman AS and Selim MA 2018. Optimization of germination, callus induction, and cell suspension culture of African locust beans *Parkia biglobosa* (Jacq.) Benth. J. of Genetic. Eng. and Biotechnol. **16**(1):191-201.

Abdel-Aty AS and Abdel-Megeed A 2015. Pesticidal activity of an isolated limonoid from *Melia azedarach* L. fruits. J. Anim. Plant Sci. **25**(2): 519-27.

Al-Khayri JM, Shamblin CE and Anderson EJ 1996. Callus induction and plant regeneration of US rice genotypes as affected by medium constituents. In Vitro Cell Dev-Pl **32**(4): 227-32.

Babu VS, Narasimhan S and Nair GM 2008. Enhanced accumulation of triterpenoids and flavonoids in cell suspension cultures of *Azadirachta indica* (A. Juss.) with an extended stationary phase. Ind. J. of Biotech. **7**: 270-272.

Balandrin MF, Klocke JA 1988. Medicinal, aromatic, and industrial materials from plants. In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 4. Medicinal and Aromatic Plants I. Springer-Verlag, Berlin, pp 3-36.

Benavides MP 1997. Growth and thiophene accumulation are influenced by sucrose, light and subculture in cell suspensions and root cultures of *Tagetes argentina*. Biocell. **21**(1): 1-11.

Bhatia P and Ashwath N 2005. Effect of medium pH on shoot regeneration from the cotyledonary explants of tomato. Biotech. **4**(1): 7-10.

Cheng H, Yu LJ, Hu QY, Chen SC and Sun YP 2006. Establishment of callus and cell suspension cultures of Corydalis saxicola bunting, a rare medicinal plant. Z. Naturfor. C. **61**(3-4): 251-256.

Cherif AO 2012. Phytochemicals components as bioactive foods. Bioactive Compounds in Phytomedicine **18**:113-24.

Choi SH, Oh CT, Kim SH, Kim YT and Jeon SH 2000. Effects of polycomb group mutations on the expression of ultrabithorax in the *Drosophila* visceral mesoderm. Mol. Cells **10**(2):156-61.

Coria C, Almiron W, Valladares G, Carpinella C, Ludueña F, Defago M and Palacios S 2008. Larvicide and oviposition deterrent effects of fruit and leaf extracts from *Melia azedarach* L. on *Aedes aegypti* (L.) (Diptera: Culicidae). Bioresour. Technol. **99**(8): 3066-70.

Dai J, Yaylayan VA, Raghavan GV and Parè JR 1999. Extraction and colorimetric determination of azadirachtin-related limonoids in neem seed kernel. J. Agric. Food. Chem. **47**(9): 3738-42.

Ding BZ, Bai SH, Wu Y and Fan XP 1981. Induction of callus and regeneration of plantlets from corm of *Crocus sativus* L. Chih Wu Hsueh Pao= Acta Bot Sin.

Ferri M, Dipalo SC, Bagni N and Tassoni A 2011. Chitosan elicits mono-glucosylated stilbene production and release in fed-batch bioreactor cultures of grape cells. Food Chemi. **124**(4): 1473-9.

Guerriero G, Berni R, Muñoz-Sanchez JA, Apone F, Abdel-Salam EM, Qahtan AA, Alatar AA, Cantini C, Cai G, Hausman JF and Siddiqui KS 2018. Production of plant secondary metabolites: examples, tips and suggestions for biotechnologists. Genes **9**(6):309.

Hammad EA, Nemer NM and Kawar NS 2000. Efficacy of chinaberry tree (*Meliaceae*) aqueous extracts and certain insecticides against the pea leaf miner (Diptera: Agromyzidae). J. Agric. Sci. **134**(4): 413-20.

Hammad EA, Zournajian H and Talhouk S 2001. Efficacy of extracts of *Melia azedarach* L. callus, leaves and fruits against adults of the sweet potato whitefly *Bemisia tabaci* (Hom., Aleyrodidae). J. Appl. Entomol. **125**(8): 483-8.

Jarvis, AP, Johnson S and Morgan ED 1998. Stability of the natural insecticide *azadirachtin* in aqueous and organic solvents. Pest. Sci. **53**(3):217-222.

Jassim SA and Naji MA 2003. Novel antiviral agents: A medicinal plant perspective. J. Appl. Microbiol. **95**(3): 412-27.

Khanavi M, Safavi M, Siavoshi F, Fallah Tafti A, Hajimahmoodi M, Hadjiakhoondi A, Rezazadeh S and Foroumadi A 2008. Evaluation of anti-helicobacter pylori activity of methanol extracts of some species of *Stachys* L. and *Melia*. L. J. Med. Plant. **4**(28): 74-80.

Mazumder ME and Rahman S 2008. Pharmacological evaluation of Bangladeshi medicinal plants for antioxidant activity. Pharm. Biol. **46**(10-11): 704-709.

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

Nasir E, Ali SI and Stewart RR 1972. An annotated catalogue of the vascular plants of West Pakistan and Kashmir/by R. R. Stewart. (Flora of West Pakistan). Karachi: Printed at Fakhri Print. Press. https://trove.nla.gov.au/version/8994146

Nhut DT, Van Le B and Van KT 2000. Somatic embryogenesis and direct shoot regeneration of rice (*Oryza sativa* L.) using thin cell layer culture of apical meristematic tissue. J. Plant. Physiol. **157**(5): 559-65.

Parr AJ 1988. Secondary products from plant cell culture. *In:* A. Mizrahi (ed), Biotechnology and Agricultur. Alan R Liss, Inc., New Yark. Page: 1-34.

Pasqua G, Monacelli B, Mulinacci N, Rinaldi S, Giaccherini C, Innocenti M and Vinceri FF 2005. The effect of growth regulators and sucrose on anthocyanin production in *Camptotheca acuminata* cell cultures. Plant. Physiol. Biochem. **43**(3): 293-8.

Potters G, Pasternak TP, Guisez Y, Palme KJ and Jansen MA 2007. Stress-induced morphogenic responses: Growing out of trouble? Trends. Plant. Sci. **12**(3): 98-105.

Prakash G, Bhojwani SS and Srivastava AK 2002. Production of azadirachtin from plant tissue culture: State of the art and future prospects. Biotech. Bioprocess. Engine. **7**(4): 185-93.

Rafiq M and Dahot MU 2010. Callus and azadirachtin related limonoids production through *in vitro* culture of neem (*Azadirachta indica* A. Juss). Afr. J. Biotechnol. 9(4): 449-453.

Samar A, Zahoor AK and Seema S 2013. Comparative efficiency of different explants for *in vitro* callus production in *Inula royleana* DC., a threatened medicinal plant growing in Kashmir Himalaya. Intr. J. Adv. Research **1**(7): 617-623

Schmidt GH, Rembold H, Ahmed AA and Breuer M 1998. Effect of *Melia azedarach* fruit extract on juvenile hormone titer and protein content in the hemolymph of two species of noctuid lepidopteran larvae [insecta: Lepidoptera: Noctuidae]. Phytoparasitica. **26**(4): 283.

Schmutterer H and Wilps HS 1995. Activity (fitness, mobility, vigor). The neem tree: *Azadichta indica* A. Juss and other meliaceous plants: Sources of unique natural products for integrated pest management, medicine, industry and other purposes. VCH. New York. 680. ISBN 3-527-30054-6.

Shekhawat KK, Rao DV and Batra A 2014. Phytomorphological overview of medicinal plant: *Melia azedarach* Linn. J. Functional. Environ. Botany. **4**(1):10-21.

Simões C, Albarello N, Castro TC and Mansur E 2012. Production of anthocyanins by plant cell and tissue culture strategies. Biotechnolo. Plant. Sec. Metab.. Bentham Science Publishers. pp. 67-86.

Sujanya S, Devi BP and Sai I 2008. *In vitro* production of azadirachtin from cell suspension cultures of *Azadirachta indica*. J. Biosciences **33**(1): 113-20.

Sundaram KM, Campbell R, Sloane L and Studens J 1995. Uptake, translocation, persistence and fate of azadirachtin in aspen plants (*Populus tremuloides* Michx.) and its effect on pestiferous two-spotted spider mite (*Tetranychus urticae* Koch). Crop Prot. **14**(5): 415-21.

Szeto SY and Wan MT 1996. Hydrolysis of azadirachtin in buffered and natural waters. J Agri. Food Chem. **44**(4): 1160-1163.

Thirumalai T, Kelumalai E, Senthilkumar B and David E 2009. Ethnobotanical study of medicinal plants used by the local people in Vellore District, Tamilnadu, India. Ethnobotanical Leaflets **10**: 1302-1311.

Ushiyama K and Hibino K 1997. Commercial production of ginseng by plant cell culture. Am. Chem. Soc. In 213th National Meeting, San Francisco, CA, USA.

Wallace RJ 2004. Antimicrobial properties of plant secondary metabolites. Proc. Nutr. Soc. **63**(4): 621-9.

Wandscheer CB, Duque JE, De Silva MA, Fukuyama Y, Wohlke JL, Adelmann J and Fontana JD 2004. Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito Aedes aegypti. Toxicon. **44**(8): 829-35.

Wang HQ, Zhong JJ and Yu JT 1997. Enhanced production of taxol in suspension cultures of *Taxus chinensis* by controlling inoculum size. Biotech. Letters **19**(4): 353-356.

Wink M 2003. Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. Phytochemistry **64**(1): 3-19.

Yang L, Wen KS, Ruan X, Zhao YX, Wei F and Wang Q 2018. Response of plant secondary metabolites to environmental factors. Molecules **23**(4): 762.

Zeng F, Wang W, Zhan Y and Xin Y 2009. Establishment of the callus and cell suspension culture of *Elaeagnus angustifolia* for the production of condensed tannins. Afr. J. Biotechnol. **8**(19): 5005-5010

(Manuscript received on 5 October, 2019; revised on 30 May, 2020)