Original Research Article

In vitro callus induction of *Catunaregam spinosa* using leaves as explant

ABSTRACT

Aims: *C. spinosa* (Family Rubiaceae) is a valuable medicinal since years ago. Callus is a source of secondary metabolites those can isolate through cell culture. A protocol was developed for callus induction of *C. spinosa* using leaf discs of as explants.

Methodology: Sterilization protocol optimized using different concentrations of Carbendazim® (0.2, 0.3 %) and Clorox (10, 15 %) exposing to different time intervals (10, 15 min). Percentage survival and contaminations were calculated. Best medium was optimized using different concentrations of 6-Benzyl Amino Purine (BAP) and Naphthalene Acetic Acid (NAA) (1.0-6.0 mg L^{-1}). Growth regulators free Murashige and Skoog (MS) medium was used as control. Completely randomized design was followed with ten replicates in each concentration. Days taken to initiate calli, morphological characteristics and mean dry weights of calli were evaluated after 3 months of incubation.

Results: Leaf discs sterilization with 0.3 % Carbendazim for 10 min, 10 % Clorox for 10 min and 70 % ethanol for 30 sec followed by two washings in sterile distilled water was found to be best sterilization protocol. It recorded lowest percentage contamination (15.05%) and highest percentage survival (86.66%) after 8 weeks. No observable changes were found in calli grown in growth regulators free MS medium. Calli growth and morphologies were significantly affected by the type of growth regulator and their concentrations in MS medium. Color of the calli varied from white opaque to yellow brown to green and the texture from foamy, loose, and friable to compact. Best medium with 1.0 mg L^{-1} BAP and 3.0 mg L^{-1} NAA produced green friable calli with 0.0969±0.01 g mean dry weight after 3 months. Some traits were found to be depended on synergetic effect of growth regulators and genotypic characteristics of explant.

Conclusion: The study influences producing calli of *C. spinosa* which would be helpful in rapid production of secondary metabolites through tissue culture compared to conventional propagation methods.

Keywords: Catunaregam spinosa, tissue culture, sterilization, plant growth regulators, callus

1. INTRODUCTION

Catunaregam spinosa (Thunb.) Tirveng is well recognized medicinal plant grows in tropical and sub-tropical countries. It possesses several pharmacological properties such as cytotoxicity, piscicidal, anti- bacterial, anti- inflammatory, hepatoprotective, insecticidal and

anthelmintic activities etc. *C. spinosa* contains diverse range of phytochemical contained with alkaloids, saponins, triterpenoids, flavonoids, cardiac and cyanogenic glycosides, tannins and volatile oils. Alkaloids are important in treatments of cancer, malaria and diabetes [1]. Further it is used 25-75 % in drug development [2]. Saponins have hypolipidemic, cytotoxic and anti-diabatic properties [3,4,5]. Likewise other compounds of *C. spinosa* also play vital roles in different activities. Crushed parts of *C. spinosa* are used in fish harvesting. Emetic activity is also widely occupied in Ayurveda medicine using to its vomit inducing effect.

Plant tissue culture is a rapid method of producing plantlets under controlled conditions. It overcomes the limitations in conventional propagation such as long term seed dormancy, lack of seed viability, delayed rooting of seedlings, constraints due to seasonal changes while expanding the potential of mass propagation. Conventional propagation methods such as seed germination, rooting of cuttings and grafting show low growth rate. This can be expected due to extended time duration taken by plants to produce high content of secondary metabolites. Root suckers are also natural asexual reproduction method reported in *C. spinosa* [6].

Pharmaceutical industry shows raised demand over secondary metabolites. This demand is not sufficiently addressed due to low production rate of metabolites in plants up to the desired level. Calli obtains from tender parts of the plants via tissue culture produce these important constituents in a significantly short period of time. Based on that calli are employed to isolate these precious compounds using cell or callus cultures as an alternative method. Ramos *et al*., (2018) used callus culture of *Ageratina pichinchensis* to produce secondary metabolites with anti-inflammatory activity [7]. Macedo *et al*., (1999) reported callus culture of *Alternanthera brasiliana* in producing antinociceptive compounds [8]. However, literatures found on *in vitro* growth of *C. spinosa* were fairly low. Further no reports were found regarding *in vitro* culture or callus induction of *C. spinosa* using leaf explants. Begum *et al*., (2003) studied on *in vitro* seed germination of *C. spinosa*. According to that maximum number of shoots (12.7) was obtained from seedlings grown in MS medium supplemented with 1.0 mg L^{-1} each of BAP and NAA [9]. Nevertheless, each species demands unique plant growth regulators and their concentrations for an optimum *in vitro* growth [10]. Thus, it is prerequisite to optimize particular level of concentrations of growth regulators in callus induction of *C. spinosa* whereas a sterilization protocol needs to be optimized beforehand. Present study intended to develop a successful sterilization protocol and to determine the best medium for *in vitro* callus induction of *C. spinosa* using leaf explants.

2. MATERIAL AND METHODS

2.1. Optimization of surface sterilization protocol

Immature leave were collected from plants grown in an open area of University of Sri Jayewardenepura, Sri Lanka. Leaves were washed with disinfectants (Dettol), 5% teepol (v/v, liquid detergent) under running tap water for 1 h. After that leaves were washed with few drops of Tween 20 for 5 mins. Under aseptic conditions sterilization protocol was tested with 0.2 and 0.3 % of Carbendazim® for 10 and 15 min. and Sodium hypochlorite (Clorox) with 10 and 15 % for 10, 15 mins. Finally the explants were washed with 70% ethanol for 30 sec. Each step was followed with two successive washings in sterile distilled water. Sterilized leaves were cut into 1.0 cm^2 pieces along midrib. Leaf discs were cultured on half MS medium [11]. Well-sealed cultured media were incubated at 25℃ and 60 % humidity. They were provided with 50 $\mu molm^{-2}s^{-1}$ white fluorescent light in 16/8 h light/ dark photoperiod. There were 15 replicates in each treatment. Percentage contamination and survival rate were determined after three months of incubation.

2.2. Determination of best medium for callus induction from leaf explants

Immature leaves from *in vivo* grown plants were used as explants. Leaf cuttings were sterilized following best surface sterilization protocol determined. Half MS medium supplemented with concentration combinations of 1.0 -6.0 mg L^{-1} NAA and BAP were tested. Medium pH was adjusted to 5.78± 0.02. Culture media were sterilized by autoclaving. Growth regulators free media were used as controls. Completely randomized design was followed with 10 replicates in each concentration. Color and texture of calli were determined by visual observations. Dry weights of calli were measured after three months of growth. Data were statistically analyzed using ANOVA at significant level of p =0.05. Medium which produced healthy flourished calli within a minimum number of days with a significantly high mean dry weight was selected as the best medium for obtaining calli of *C. spinosa* from leaf discs explants.

3. RESULTS AND DISCUSSION

3.1. Optimization of surface sterilization protocol

Protocol which exhibited lowest percentage contamination and highest percentage survival selected as best sterilization protocol. Best sterilization protocol found to be the leaf discs washing with 0.3 % carbendazim for 10 minutes, 10 % Clorox for 10 minutes and 70 % ethanol for 30 sec followed by successive washing twice with sterile distilled water. It exhibited lowest percentage contamination (15.05 %) and highest percentage survival (86.66 %) after eight weeks of incubation (Table 1). At 15 % concentration of Clorox soft tissues of leaf discs were damaged leading them to necrosis within first week of incubation. Explants disinfected with 0.3 % carbendazim for 15 mins recorded lowest contamination percentages of 13.33 %. However, exposure to a high concentration of fungicides and bleaching agents for a long period of time can lead growing mutations and affect the regeneration capacity [12]. This trait was observed when leaf discs exposed to15 % carbendazim for 15 min and 15 % Clorox for 15 min where the percentage survival was low (46.66 %) while having low percentage contamination (13.33 %). Thus 0.3 % carbendazim for 10 mins and 10 % Clorox for 10 min were selected as the part of best sterilization protocol.

Table 1. Percentage survival and percentage contaminations of different treatments used for optimizing surface sterilization protocol of *C. spinosa* leaf explants

Treatment code	Clorox		Carbendazim		% survival	% contamination
	Concentration (v/v)	Exposure time (mins)	Concentration (w/v)	Exposure time (mins)		
T1	10	10	0.2	15	40.00	60.00
T2	10	10	0.2	10	13.33	86.66
T3	10	10	0.3	15	80.00	20.00
T4	**10**	**10**	**0.3**	**10**	**86.66**	**15.05**
T5	10	15	0.2	10	73.33	26.66
T6	10	15	0.2	15	66.66	33.33
T7	15	15	0.2	10	53.33	46.66
T8	15	15	0.2	15	46.66	66.66
T9	15	10	0.3	10	73.33	26.66

T10	15	10	0.3	15	66.66	20.00
T11	15	15	0.3	10	53.33	40.00
T12	15	15	0.3	15	46.66	13.33

3.2. Determination of best medium for callus induction from leaf explants

Callus induction and growth rate varied among the tested concentrations. Calli grown in plant growth regulators free MS medium (control) showed no observable changes over first 3 weeks (Plate 1a). Media supplemented with plant growth regulators took minimum 12.2±0.79 mean number of days for callus initiation. It proved the significant role of plant growth regulators in callus induction and their growth. Cells regeneration process was observed with inwardly rolled and swelled leaf discs with wavy cutting edges after two weeks of incubation (Plate 1b). After three months of growth healthy, flourished calli were observed with protuberance appearance on calli grown in media supplemented with plant growth regulators (Plate 1c).

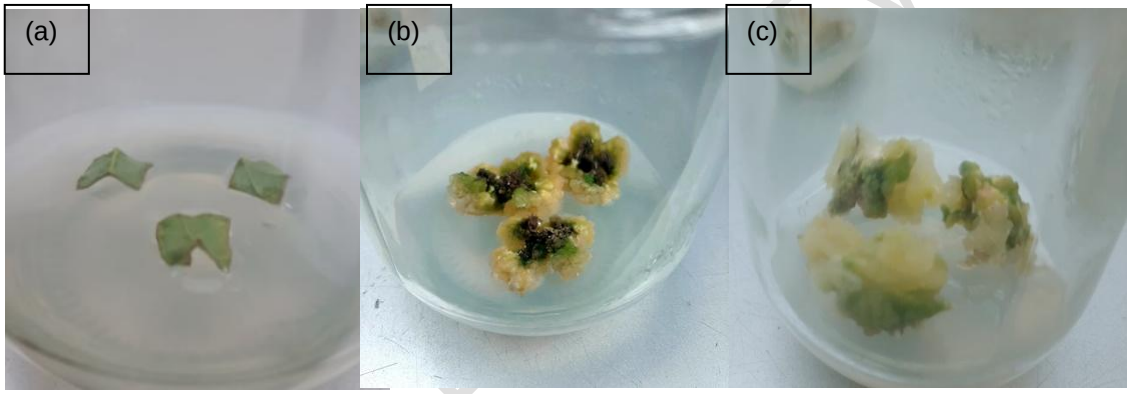

Plate 1: Calli grown in plant growth regulators (a) free MS medium after three weeks (b) added medium after two weeks (c) added medium after three months of incubation

Morphology and callus inductions have significantly influenced by both individual and combined concentrations of BAP and NAA. Color of the calli varied from white opaque to yellow brown to green (Plate 2). Texture was friable, foamy, loose or compacted (Plate 3).

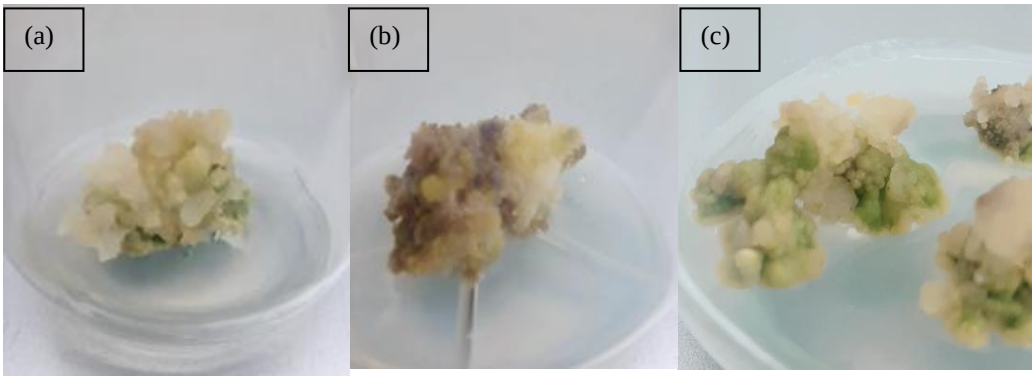

Plate 2: Color morphology of calli grown in MS media treated with different concentrations of BAP and NAA (a) white opaque (b) yellow brown (c) green

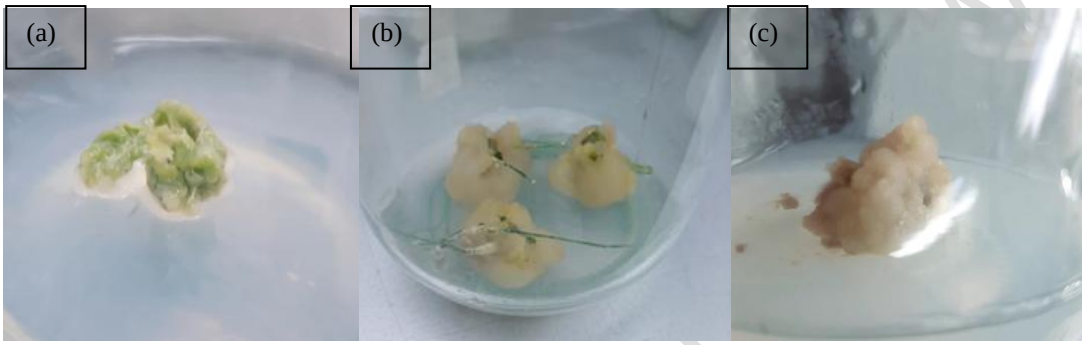

Plate 3: Texture morphology of calli grown in MS media supplemented with different concentrations of BAP and NAA (a) compact (b) foamy (c) friable

Concentration dependent callus growth is shown in Table 2.

Table 2. Effect of different concentrations of BAP and NAA on mean no. of days and mean dry weight of *C. spinosa* leaf explants after three months of growth

Culture tag	Concentration		Mean no. of days taken to initiate callus ± SD (Days)	Mean dry weight (g)
	BAP	NAA		
C0	0	0	25.4±0.96	0.013±0.005
C1	1	0	18.5±0.52	0.014±0.005
C2	2	0	18.4±0.51	0.018±0.010
C3	3	0	17.8±0.78	0.021±0.005
C4	4	0	17.1±0.74	0.026±0.01
C5	5	0	17.2±0.78	0.030±0.006
C6	6	0	17.1±0.74	0.034±0.006
C7	0	1	15.9±0.73	0.042±0.007
C8	1	1	16.8±0.78	0.043±0.032
C9	2	1	16.0±0.82	0.057±0.009
C10	3	1	16.2±1.03	0.075±0.007
C11	4	1	16.4±0.51	0.069±0.011
C12	5	1	16.3±1.06	0.079±0.010
C13	6	1	16.0±0.94	0.074±0.014
C14	0	2	14.3±0.95	0.086±0.052

C15	1	2	16.6±0.69	0.084±0.027
C16	2	2	13.2±0.78	0.091±0.045
C17	3	2	12.6±0.69	0.094±0.045
C18	4	2	12.8±1.03	0.090±0.014
C19	5	2	12.2±0.79	0.090±0.011
C20	6	2	13.2±0.79	0.096±0.037
C21	0	3	12.8±0.78	0.086±0.020
C22	**1**	**3**	**13.2±1.03**	**0.096±0.019**
C23	2	3	13.3±1.06	0.073±0.010
C24	3	3	13.7±0.97	0.084±0.022
C25	4	3	14.7±0.67	0.074±0.018
C26	5	3	13.5±1.19	0.074±0.014
C27	6	3	13.0±0.70	0.078±0.009
C28	0	4	13.2±0.78	0.046±0.008
C29	1	4	14.2±0.78	0.074±0.007
C30	2	4	14.0±0.94	0.081±0.024
C31	3	4	14.7±0.95	0.088±0.009
C32	4	4	15.0±1.05	0.066±0.020
C33	5	4	15.4±1.33	0.051±0.019
C34	6	4	16.9±0.88	0.073±0.010
C35	0	5	15.7±0.67	0.070±0.009
C36	1	5	15.9±0.73	0.083±0.010
C37	2	5	15.8±0.79	0.074±0.007
C38	3	5	15.9±0.74	0.068±0.009
C39	4	5	16.0±0.81	0.083±0.015
C40	5	5	14.6±0.84	0.070±0.005
C41	6	5	16.2±0.63	0.064±0.014
C42	0	6	16.6±0.69	0.076±0.010
C43	1	6	15.9±0.74	0.053±0.007
C44	2	6	16.0±0.81	0.045±0.007
C45	3	6	17.0±0.82	0.032±0.009
C46	4	6	15.7±0.67	0.033±0.006
C47	5	6	17.1±0.73	0.033±0.009
C48	6	6	12.6±0.69	0.044±0.006

Callus induction and growth varied with single or combined use of BAP and NAA. NAA alone initiated the callus induction. Both callus induction and growth rate were low when BAP alone used. High concentrations of NAA inhibited the callus induction where it recorded relatively high mean number of days to initiate calli. MS media supplemented with 1.0, 2.0 and 3.0 mg L^{-1} NAA concentrations alone only spent 15.9±0.73, 14.3±0.95 and 12.8±0.78 mean number of days for callus induction whereas no significant prompt callus induction was observed at high concentrations of NAA alone (4.0, 5.0 and 6.0 mg L^{-1}). Greenish compact calli were observed at low concentrations of NAA independent of the BAP concentration. Their mean dry weights were <0.04g and mean number of days taken to initiate calli were between 18.5 - 17.1 days. Calli grown in MS media

supplemented with 2.0 – 3.0 mg L^{-1} NAA concentrations (C16-C27) recorded lowest mean number of days (12.2- 14.7 days) for callus induction compared to all other combinations. They produced flourished, friable calli with high mean dry calli weights. MS media treated with 4.0 – 6.0 mgL^{-1} concentrations of NAA (C29-C48) produced more whitish and loosen calli with 14.0 -17.1 days mean number of days. There were some instances followed opposite of this scenario where treatments with high concentration of NAA recorded low mean number of days and vice versa. MS media supplemented with 5.0 mg L^{-1} (C40) and 6.0 mg L^{-1} (C48) NAA only spent 12.6±0.69 and 12.8±0.78 days respectively and MS medium treated with 1.0 mg L^{-1} BAP and 2.0 mg L^{-1} NAA recorded relative high mean number of days (15.5±0.92 days) to produce calli. These can be probably caused by effect of genotypic characteristics of explants on callus induction [14].

BAP started to form new cells by stimulating cell division at the presence of optimal concentration of NAA. New cell formation expanded the leaf explants to form calli with optimum weights. Based on that we can predict the possible reason causing high mean dry weights of calli produced in media treated with 2.0-3.0 mg L^{-1} of NAA along increasing concentration of BAP. However, Ahmad and Spoor, (1999) observed enhanced callus growth whenever the BAP and NAA concentrations are balanced. They revealed the maximum callus growth of *Brassica oleraces* L. was at 1.0 mg L^{-1} each BAP and NAA concentrations [15].

According to Gaspar *et al*., (1996) cultures only treated with cytokinin (BAP) did not yield any embryonic calli but mostly towards compact calli. Cultures treated with combined formulations of auxin and cytokinin stimulated the callus induction and rapid subsequent growth [13]. Lee *et al*., (2010) mentioned the role of cytokinin in cell division of developing calli and role of auxin in both of cell division and cell growth [16]. Synergetic effect of plant growth regulators remarkably affects either enhanced or suppressed growth of calli [17]. Combined formulations of BAP and NAA produced various morphological changes on calli. In present study, addition of BAP along with NAA produced more flourished and healthy calli rather small and compact calli.

At high concentrations of NAA and BAP recorded delayed growth and callus induction recording low mean dry weights and high mean number of days for callus induction. This trait can be attributed to the inhibitory effect of higher concentrations of plant growth regulators. Similarly, Dar et al., (2021) observed decreased production of calli obtained from leaf explants of *Atropa acuminata* after 1 mg L^{-1} each of BAP and NAA [18]. Mostafiz and Wagiran, (2018) also observed retarded growth of calli at threshold concentration of 2, 4-D (3.0 mgL^{-1}) in a study of *in vitro* callus induction of *Indica rice* species [19]. Liu *et al*., (2018) mentioned higher concentrations of BAP and NAA can produce foamy and soft calli with low weights [20]. In our study also at higher concentration of BAP and NAA (4.0, 5.0 and 6.0 mgL^{-1}) formy and loosened calli were observed with soft texture.

Based on the overall growth of calli, medium supplemented with 1.0 mg L^{-1} BAP and 3.0 mg L^{-1} NAA (C22) was the best medium for callus induction of *C. spinosa* using leaf explants. Calli grown in this medium recorded highest mean dry weight (0.0969±0.01 g) and low mean number of days (13.2±1.03 days) to produce pronounced, healthy, green and friable calli.

Ikeuchi *et al*., (2013) mentioned intermediate ratio of auxin to cytokinin promotes callus formation and high ratio of auxin to cytokinin and vice versa promote root and shoots respectively [21]. However according to Flick *et al*., (1983) high concentration of auxin to

low concentration of cytokinin is typical combination to promote mass cell proliferation [22]. The same scenario has proven in a study of callus induction of *Coffea arabica* L. using leaf cultures. The prominent friable calli have formed in 2.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP concentrations [14]. In our study also best medium was contained with high concentration of auxin (3.0 mgL^{-1}) to low concentration of cytokinin (1.0 mgL^{-1}) ratio.

4. CONCLUSION

Callus production is possible in *C. spinosa* as a plant with significant phytochemicals. Best sterilization protocol for explants grown in open environment is disinfecting with 0.3 % carbendazim for 10 mins, 10 % Clorox for 10 mins. and 70 % ethanol for 30 sec. which recorded lowest contamination and highest survival percentages compared to other combinations. Half MS medium supplemented with 3.0 mgL^{-1} of BAP and 1.0 mgL^{-1} NAA was best medium for callus induction of *C. spinosa* from leaf discs. Callus morphology and mean dry weights of calli were dependent on type of plant growth regulator and their concentrations. The study proves potential of using leaves of *C. spinosa* for callus production which would be useful in isolation of potential secondary metabolites.

REFERENCES

1. Ain QU, Khan H, Mubarak MS and Pervaiz A. Plant alkaloids as antiplatelet agent: Drugs of the Future in the Light of Recent Developments. Frontier Pharmacology. 2016; 7: 292. Doi: 10.3389/fphar.2016.00292.
2. Khan H. Medicinal plants need biological screening: a future treasure as therapeutic agents. Biology and Medicine. 2014b; 6:e110. Doi: 10.4172/0974-8369.1000e110.
3. Elekofehinti OO, Iwaloye O, Olawale F and Ariyo EO. Saponins in cancer treatment: Current progress and future prospects. Pathophysiology. 2021; 28(2): 250-272. Doi:10.3390/pathophysiology28020017.
4. Ejelonu OC, Elekofehinti OO and Adanlawo IG. *Tithonia diversifolia* saponin-blood lipid interaction and its influence on immune system of normal wistar rats. Biomedicine & Pharmacotherapy. 2017; 87: 589-595. Doi:10.1016/j.biopha.2017.01.017. ISSN 1950-6007. PMID 28086134.
5. Kim JJJ, Xiao H, Tan Y, Wang ZZ, Seale JP and Qu X. The Effects and mechanism of saponins of *Panax notoginseng* on glucose metabolism in 3T3-L1 cells. The American Journal of Chinese Medicine. 2009; 37(6): 1179–1189. Doi:10.1142/S0192415X09007582. ISSN 0192-415X. PMID 19938225.
6. Ashton R. The incredible pomegranate. Plant and Fruit, ed. Baer B and Silverstein D. United States of America: ThirdMillennium Publishing. 2006;162.
7. Ramos MS, Bahena MS, 1, Estrada AR, Antonio, AB, Sosa FC, Christen JG, Fernández, JJA, Arango, IP and Alvarez,L. Establishment and phytochemical analysis of a callus culture from *Ageratina pichinchensis* (Asteraceae) and its anti-inflammatory activity. Molecules. 2018; 23: 1258.
8. Macedo AF, Barbosa NC, Esquibel MA, Souza, MM and Filho VC. Pharmacological and phytochemical studies of callus culture extracts from *Alternanthera brasiliana*. Pharmazie. 1999; 54(10):776-7.
9. Begum F, Islam KMD, Paul RN, Mehedi M and Rani S. *In vitro* propagation of emetic nut *Randia dumetorum* (Lamb.). Indian Journal of Experimental Biology. 2003; 41: 1479-1481.

10. Dunwell JM. *In vitro* regeneration from excised leaf discs of three Brassica species. Journal of Experimental Botany. 1981; 32: 789-799.
11. Murashige T and Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiology. 1962;15: 473–497.
12. Yildiz M, Fatih OS., Cansu TK and Tuna E. The effect of sodium hypochlorite solutions on the viability and *in vitro* regeneration capacity of the tissue, The Natural Products Journal. 2012; 2(4) https://dx.doi.org/10.2174/2210315511202040328.
13. Gaspar T, Kevers C, Penel C, Greppin H and Reid DM. Plant hormones and plant growth regulators in plant tissue culture. *In Vitro* Cell.Development.Biology Plant. 1996; 32**:** 272–289 https://doi.org/10.1007/BF02822700
14. Irene WM, Alumiro HL, Asava KK, Agwanda CO and Anami SE. Effects of genotype and plant growth regulators on callus induction in leaf cultures of *Coffea arabica* L. F1 Hybrid. Journal of Plant Biochemistry & Physiology. 2019; 7 (2); 236.
15. Ahmad S and Spoor W. Effects of NAA and BAP on callus culture and plant regeneration in Curly Kale (*Brassica oleraces* L.). Pakistan Journal of Biological Sciences. 1999; 2 (1): 109-112.
16. Lee Y, Lee DE, Lee HS, Kim SK, Lee WS, Kim SW and Kimet MW. Influence of auxins, cytokinins, and nitrogen on production of rutin from callus and adventitious roots of the white mulberry tree (*Morus alba* L.). Plant Cell, Tissue and Organ Culture (PCTOC). 2010; 105(1): 9-19.
17. Varshney A, Anis M and Aref IM. Potential role of cytokinin–auxin synergism, antioxidant enzymes activities and appraisal of genetic stability in *Dianthus caryophyllus* L.—an important cut flower crop. *In Vitro* Cell Development Biology Plant. 2013; 49**:** 166–174 https://doi.org/10.1007/s11627-012-9474-8.
18. Dar SA, Nawchoo IA, Tyub S and Kamili AN. Effect of plant growth regulators on *in vitro* induction and maintenance of callus from leaf and root explants of *Atropa acuminata* Royal ex Lindl. Biotechnology Reports. 2021. 32.
19. Mostafiz SB and Wagiran A. Efficient callus induction and regeneration in selected *Indica* rice. Agronomy, 2018. 8(5). Doi:10.3390/agronomy8050077.
20. Liu J, Feng H, Ma Y, Zhang L, Han H and Huang X. Effects of different plant hormones on callus induction and plant regeneration of miniature roses (*Rosa hybrida* L.). Horticulture International Journal. 2018; 2(4): 201-206.
21. Ikeuchi M, Sugimoto K and Iwase A. Plant callus: Mechanisms of induction and repression. The Plant Cell. 2013; 25: 3159–3173.
22. Flick CE, Evans DA and Sharp WR. Organogenesis. In: Handbook of Plant Cell Culture, Techniques for Propagation and Breeding. Editors; Evans DA, Sharp WR, Ammirato PV and Yamada Y. Macmillan Publishing Company, New York, USA.: 1983; 1: 13-81.