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Effect of plant growth regulators on *in vitro* induction and maintenance of callus from leaf and root explants of *Atropa acuminata* Royal ex Lindl

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ABSTRACT

Atropa acuminata, an important medicinal plant belonging to family Solanaceae is under tremendous threat of extinction in its natural habitat due to the overexploitation by pharmaceutical industries. Present study is an attempt of establishing callus cultures of this important medicinal plant as callus has considerable potential as an alternative for production of secondary metabolites for industrial use, hence reducing pressure on natural populations.

Callus cultures were established from leaf and root explants of *Atropa acuminate*. Murashige and Skoog (MS) media containing different concentration and combinations of 6-Benzyl Amino Purine (BAP), Naphthalene Acetic Acid (NAA), Kinetin (Kn) and 2,4- Dichloropheoxyacetic acid (2,4- D) were used for callus induction. Different phytohormonal combinations resulted in different types and degrees of callus. The combination of BAP and NAA on MS media supplemented with 0.5 mg/l BAP in combination with 1.0 mg/l NAA, was found to be the most efficient for in vitro callus development from root explants and from leaf explants most effective combination and concentration was 1 mg/l of both BAP and NAA. The maximum mean fresh weight of callus formed using root explants was 33.13 mg per explant and maximum fresh weight obtained from leaf explants was 22.14 mg per explants.

Abbreviations

MS:	Murashige and Skoog basal medium
BAP:	6-benzylaminopurine
NAA:	α-Naphthalene acetic acid
Kn:	kinetin
MFW:	Mean Fresh Weight
MDW:	Mean Dry Weight
2, 4- D:	2,4- Dichloropheoxyacetic acid

1. Introduction

Atropa acuminata commonly known as Indian Belladonna is a genus of the herbaceous perennial plant belonging to the family of Solanaceae and is endemic to northern Pakistan, Kashmir, and India [1–3]. It is a tall, erect annual herb growing up to 2 m in height, woody on the bottom, branched on the top, and tillers from the base. The plant is an important source of alkaloids such as Atropine and scopolamine. The plant is an important source of tropane alkaloids such as hyoscyamine atropine and scopolamine, which possess the anticholinergic activity and therapeutic uses in the fields of ophthalmology, cardiology, and gastroenterology [4,5]. The leaves and roots have traditionally been used as relaxants, diuretics, mydriatics, opioids, and sedatives [6]. In the search for alternatives to the production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [7–8]. Type of explant and plant growth regulators play an important role in the initiation of callus because each explant requires a particular concentration of exogenous and endogenous hormones for callus production under in vitro conditions [9–11]. The initiation of multiple shoots from callus also depends upon the type of explant and growth regulators used [[12]]. In the present study, an attempt has been made to establish callus cultures using in vitro raised leaf and root explants of *A. acuminata* under the influence of different combinations and concentrations of BAP, NAA, Kn, and 2,4-D. The callus obtained can be used

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for the extraction of secondary metabolites for drug formation without scarifying the whole plant.

2. Materials and methods

2.1. Plant material

Atropa acuminata plant was collected during flowering stage at the site of its natural habitat at an altitude of (34° 15'N and 74° 25'E) from Afarwath area of Kashmir Himalaya (July 2020). A voucher sample was submitted to KASH herbarium under specimen no. 2862- KASH herbarium, University of Kashmir.

2.2. Preparation of medium

Medium used for culturing of explants was Murashige & Skoog's (MS) (1962) basal medium which was supplemented with 3% sucrose. Various concentrations and combinations of BAP, NAA, Kn and 2, 4-D were added to the MS medium. pH of the medium was adjusted between 5.5–5.8 and 0.8% agar was used as jelling agent. The medium after dispensing in suitable culture vials was autoclaved for 20–25 min at a pressure of 15 lb and at a temperature of 121 °C. The cultures were maintained at 23± 5 °C with 55–65% RH and exposed to 16 hr light period using cool fluorescent (3000 lux) tubes.

2.3. Surface sterilization of seeds

All the seeds were surface sterilized in 0.02% $HgCl_2$ solution for 12 min. The explants were properly rinsed with double distilled autoclaved water to remove traces of $HgCl_2$.

2.4. Seed germination

The sterilized seeds were inoculated on MS basal media (Media without plant growth hormones) and incubated in culture room under controlled conditions.

2.5. Callus production

For callus production in vitro raised leaf and root explants were inoculated on MS media augmented with different concentrations of BAP, NAA, Kn and 2,4-D. MS basal medium was used as control. The cultures were incubated in culture room and results were recorded after every week.

3. Results

3.1. Callus formation from leaf explants

To obtain callus from leaf explants, three combinations of phytohormones were used. One was combination of MS media +BAP + NAA and other was MS media + Kn + 2, 4-D and the third one included MS + 2, 4-*D* + NAA (Table 1-3). In leaf explants callus induction was obtained on all the three combinations used. But the best results in form of amount of callus obtained were on MS medium augmented with different concentrations and combination of BAP and NAA (Table 1). Out of all combinations and concentrations used maximum amount of callus obtained from leaf explants was 22. 14 mg fresh weight on concentration of 1 mg/l of both BAP and NAA. On increasing the further concentration of BAP and NAA there was decrease in amount of callus produced. So the concentration of 1 mg/l of both BAP and NAA proved to be the threshold concentration. When MS media was augmented with Kn + 2,4-D combination maximum amount of callus obtained is 6.24 mg fresh weight on 0.5 mg/l concentration of both Kn and 2,4-D (Table 2). While using the combination of 2,4-D and NAA maximum amount of callus obtained was 5.27 mg fresh weight on combination of 0.5 mg/l of 2,4-D and 1 mg/l of NAA (Table 3). In the preset study, BAP and NAA combination was most favorable for production of callus comparatively in MS media without any hormones no callus was obtained.

Table 1
Effect of BAP and NAA on callus induction from leaf explants of *A. acuminata* after three months.

Growth regulators Concentration (mg/l) BAP	NAA	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0	0			
0.5	0.5	100% a	0.58ab	14.26c
0.5	1	100% a	0.61ab	12.03e
0.5	1.5	100% a	0.45c	11.34f
0.5	2	100% a	0.47c	14.21c
0.5	2.5	100% a	0.51 c	16.14 c
0.5	3	100% a	0.56 c	16.58 c
1	1	100% a	0.74a	22.14a
1.5	1	100% a	0.68b	16.18d
2	1	100% a	0.60c	14.06c
2.5	1	100% a	0.57c	16.57c
3	1	100% a	0.59c	21.09c

Table 2
Effect of Kn and 2,4-D on callus induction from leaf explants of *A. acuminata* after three months.

Growth regulators Concentration (mg/l) 2,4-D	Kn	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0. 5	0. 5	100% a	0.25 b	6.34
1	0.5	100% a	0.14 c	4.13 d
1.5	0.5	100% a	0.09 d	2. 26 e
2	0.5	100% a	0.05 d	1.10 e
0.5	1	100% a	0.33 a	6.16 a
1	1	100% a	0.21 b	4.22
1.5	1	100% a	0.17 c	3.06 c
2	1	100% a	0.11 c	2.15 c

3.2. Callus formation from root explants

In case of root explants again all the three combinations used showed callus formation (Table 4–6). The best result in terms of amount of callus obtained i.e. 33. 13 mg fresh weight was again on BAP + NAA combination but here the concentration of BAP used is 0.5 mg/l and that of NAA is 1 mg/l. On increasing concentration of BAP and NAA there was decrease in quantity of callus produced. The maximum amount of callus obtained on Kn + 2,4-D is 7.04 mg of fresh weight on using 0.5 mg/l of both phytohormones. On using combination of 2,4-D and NAA callus production was observed comparatively in less quantity. In this

Table 3
Effect of 2, 4-D and NAA on callus initiation from leaf explants of *A. acuminata* after three months.

Growth regulators concentration (mg/l) 2,4-D	NAA	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0	0	0b	0 d	0 g
0.5	0.5	100% a	0.19 c	4.11 c
1	0.5	100% a	0.13 c	2.19 e
2	0.5	100% a	0.029 d	1.05 f
0.5	1	100% a	0.23 a	5.27 a
1	1	100% a	0. 13c	2.12 d
2	1	0 b	0 d	0 g

Table 4
Effect of BAP and NAA on callus induction from root explants of *A. acuminata* after three months.

Growth regulators concentration (mg/l) BAP	NAA	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0	0	0%c	0 h	0 j
0.5	0.5	100% a	0.81 b	27.13 b
0.5	1	100% a	0.74 a	33.13 a
0.5	1.5	100% a	0.68 e	19.32 g
0.5	2	100% a	0.49 e	16.19 g
0.5	2.5	100% a	0.41 e	17.15 g
0.5	3	100% a	0.52 d	22.14 e
1	1	100% a	0.85 c	26.12 c
1.5	1	100% a	0.72 d	22.56 c
2	1	100% a	0.63 d	18.21 d
2.5	1	100% a	0.54 d	15.11d
3	1	100% a	0.51 d	12.32 f

Table 5
Effect of KIN and 2,4-D on callus induction from root explants of *A. acuminata* after three months.

Growth regulators concentration (mg/l) 2,4-D	Kn	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0	0	0 c	0 b	0 g
0.5	0.5	100% a	0.27 a	7.04 a
1	0.5	100% a	0.20 a	3.12 bc
2	0.5	85% ab	0.20 a	4.89 b
0.5	1	100% a	0.26 a	4.65 cd
1	1	55% b	0.12 b	3.31 de
2	1	55% b	0.12 b	1.92 ef

Table 6
Effect of 2,4-D and NAA on callus formation from root explants of *A. acuminata* after three months.

Growth regulators concentration (mg/l) 2,4-D	NAA	% of callus formation	MDW of callus (mg/ Exp.)	MFW of callus (mg/ Exp.)
0	0	0 b	0.07 b	0 c
0.5	1	75% a	0.16 a	4.10 a
1	1	100% a	0.06 b	1.25 b
2	1	0% b	0 d	0 c

combination maximum amount of callus obtained was only 4.10 mg of fresh weight on using 0.5 mg/l of 2,4-D and 1 mg/l of NAA. So callus was obtained on all the three combinations but the amount of callus obtained on BAP + NAA combination is far higher than on other concentrations.

3.3. Callus characteristics

The calli formed were primarily compact, soft and vitreous, with color varying from green, brown to white and varied with different concentrations of PGR combinations used. White and green calli were obtained from leaf explants; white callus had friable texture (Fig. 1C) wshilst green callus has a compact texture (Fig 1. D). In root explants, callus was yellowish in color with a compact texture (Fig. 1E &F).

4. Discussion

Plant tissue culture plays a crucial role in the manipulation of plants for better performance [13]. Plant cell cultures, particularly callus cultures, have a wide range of physiological characteristics. Because of their heterogeneous character, developing cost-effective natural product manufacturing platforms demands the selection of high-yielding cell lines [14, 15].

In response to both biotic and abiotic stress plants develop unorganized cell masses and generate the secondary metabolites that are released as defense responses [16]. Secondary metabolites not only reveal protective functions, but also possess medicinal value for human beings and callus cultures being produced under stress conditions represent interesting sources for the easy and scalable production of secondary metabolites [17].

Since the discovery that the combination of two growth-promoting hormones, auxin and cytokinin, induces callus from plant explants in vitro, this experimental system has been used extensively [16]. Role of phytohormones in the plant tissue culture including callus induction and differentiation of various calluses is well documented. Auxin and cytokinin levels, both endogenous and exogenous, play a role in callus formation in a variety of plant species. In general, an intermediary percentage of auxin and cytokinin enhances callus induction, while a high percentage of auxin-to-cytokinin or cytokinin-to-auxin stimulates root and shoot renewal, respectively [18]. The use of cytokinins is believed to influence callus formation by lowering cell wall lignifications and so increasing callus initiation and development in vitro. It has been found that callus growth usually begins on the explants cut surface and progresses to cover the whole explant [19]. Since several studies, have shown that combining cytokinins and auxins generated superior outcomes than using either auxins or cytokinins alone [20, 21]. Hence, the present study was aimed to establish a suitable combination of BAP, NAA, kinetin, and 2,4-D to promote callus induction in *Atropa acuminata*.

In the present study auxin and cytokinin combination was used for the induction of callus. The combinations used were BAP + NAA, NAA + 2,4-D and Kn + 2,4-D. The explants used included in vitro raised root and leaf explants. Among all the combinations and concentrations used maximum amount of callus was obtained on root explants cultured on MS media supplemented with BAP+ NAA followed by leaf explants on same combination of auxin and cytokinin. As far as concentration is considered lower concentration of both the phytohormones proved to be critical for callus induction and on increasing concentration there was significant decrease in amount of callus produced. So irrespective of explants auxin and cytokinin ratio and type seems to play a role in induction of callus. Similarly some other workers like [22–27] have also obtained maximum callus on same phytohormonal combination. MS medium augmented with BAP and NAA also showed significant response for callus induction from leaf explants of *Withania somnifera* [28]. Our findings are also in accord with the previous findings of [29] where they reported the formation of compact callus of *Annona muricata* at a concentration of 2 mg/l, BAP+0.2 mg/l, NAA. Similarly, [20] reported callus induction on MS medium containing 2.5 mg /l NAA + 2.0 mg /l BAP. The decreased amount of callus produced on higher concentrations is attributed to the inhibitory effect of phytohormones when applied in higher concentrations. On using other two combinations Kn+2,4-D and NAA + 2,4-D comparatively less amount of callus was obtained. This is in contrast to many studies where 2,4-D was responsible for production of higher amount of callus in other plants like rice [30] .The lower amount of callus produced using Kn and 2,4-D in our study may be due to the endogenous presence of phytohormones with the result the critical ratio required for more callus formation is not attained.

5. Conclusion

In the present study, in vitro callus production was studied on leaf and root explants of *A. acuminata*. Callus was initiated on all concentrations and combinations but MS media augmented with BAP + NAA combination of Plant growth regulators proved to be best for production

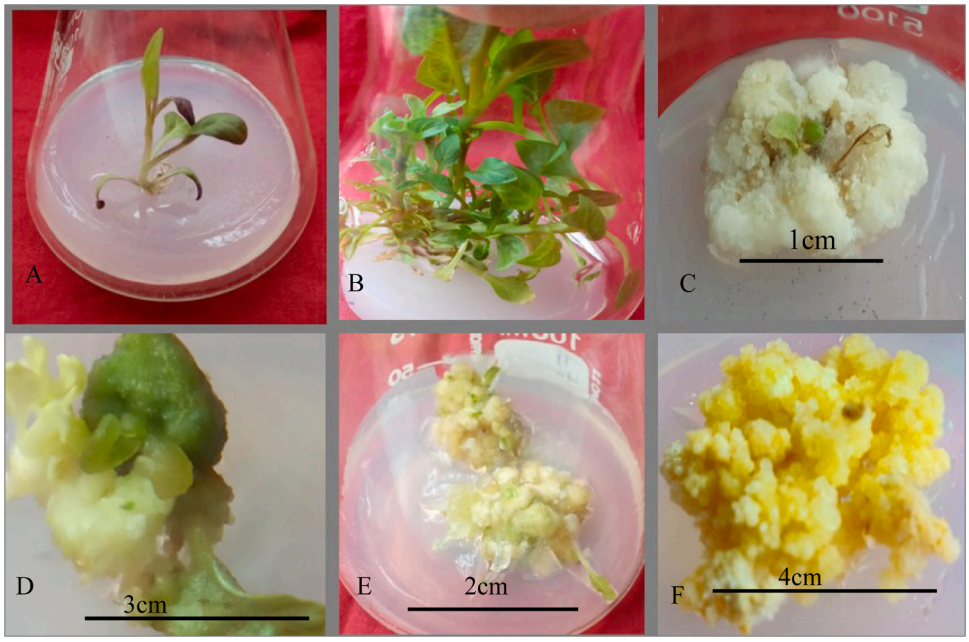

Fig. 1. A: Seed germination on MS basal medium. B: 45 days old complete seedling. C: Callus from leaf explants on BAP 1 mg/l and NAA 0.5 mg/l . D: Callus from leaf explants on BAP 1 mg/l and NAA 1 mg/l. E: Callus from root explants on BAP 0.5 mg/l and NAA 1 mg/l . F: Callus from root explants on BAP 1 mg/l and 1 mg/l NAA .

of callus in both explants. In callus production, root explants proved to be better explants as more amount of callus was obtained on root explants as compared to leaf explants. The maximum amount of callus was obtained on lower concentrations of phytohormones and higher concentrations of phytohormones proved to be inhibitory for both explants.

CRediT authorship contribution statement

Shabeer Ahmad Dar: Formal analysis, Writing – original draft. **Irshad Ahmad Nawchoo:** Formal analysis, Supervision. **Sumira Tyub:** Writing – review & editing, Formal analysis, Data curation. **Azra Nahaid Kamili:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declared that they have no conflict of interest related with the publication of this article.

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References

[1] M.K. Kaul, Medicinal Plants of Kashmir and Ladakh: Temperate and Cold Arid Himalaya, Indus publishing, 1997.
[2] G. Mehraj, et al., Patterns of alien plant diversity in the urban landscapes of global biodiversity hotspots: a case study from the Himalayas, Biodivers. Conserv. 27 (2018) 1055–1072.
[3] Mehraj, G., Khuroo, A.A., Muzafar, I., Rashid, I. & Malik, A.H. (2017). An updated taxonomic inventory of flora of Srinagar City (Kashmir Himalaya) India, using herbarium reconstruction approach. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 1–7.
[4] Cardillo, A.B., Rodriguez Talou, J., & Giulietti, A. (2016). M. establishment, culture, and scale-up of Brugmansia candida hairy roots for the production of tropane alkaloids. Protocols For In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, 2nd Edition, 173–186.
[5] L. Kursinszki, H. Hank, I. László, E. Szoke, Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed-phase high-performance liquid chromatography, J. Chromatogr. 1091 (2005) 32–39.
[6] M. Vanisree, C.Y. Lee, S.F. Lo, S.M. Nalawade, C.Y. Lin, H., S. Tsay, Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures, Bot. Bull. Acad. Sin. 45 (2004) 1–22.
[7] G.A. Ravishankar, S. Ramachandra Rao, Biotechnological production of phyto-pharmaceuticals, J. Biochem. Mol. Biol. Biophys 4 (2000) 73–102.
[8] M. Sharma, A. Sharm, A. Kumar, S.K. Basu, Enhancement of secondary metabolites in cultured plant cells through stress stimulus, American J. Pl. Physiol 6 (2) (2011) 50–71.
[9] R. Li, D.W. Reed, E. Liu, J. Nowak, L.E. Pelcher, J.E. Page, P.S. Covello, Functional genomics analysis of alkaloid biosynthesis in Hyoscyamus nigerreveals cytochrome P450 involved in littorine rearrangement, Chem. Biol. 13 (2006) 513–520.
[10] T. Murashige, F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15 (1962) 473–497.
[11] Oto, G.H., Ozdemir, B., Yaren, Y., Yetkin, A., Tas, & Tantitanir, P. (2013). Antinociceptive activity of methanol extract of Hyoscyamus reticulatusL. in mice. Am. J. Phytomedicine. Clin. Ther, 177–123.
[12] M. Rasheed, M.J. Jaskani, M. Rasheed, M.S. Iqbal, S.Z.U. Hasan, R. Rafique, S. Mushta, M. Iqbal, Regeneration of plantlets from various explants of tetraploid water melon, J. Bio. Agri & Healtcare 3 (1) (2013) 27–29.
[13] S. Adrian, S. Nigel, F. Mark, Plant biotechnology- the genetic Manipulation of Plant, Oxford university press, 2008.
[14] V. Mulabagal, H.S. Tsay, Plant cell cultures—An alternative and efficient source for the production of biologically important secondary metabolites, Int. J. Appl. Sci. En. 2 (2004) 29–48 [Google Scholar].
[15] M. Ochoa-Villarreal, S. Howat, S. Hong, M.O. Jang, Y.W. Jin, E.K. Lee, G.J. Loake, Plant cell culture strategies for the production of natural products, BMB *Re.* 49 (2016) 149–158 [Google Scholar].
[16] I. Momoko, S. Keiko, I. Akira, Plant callus: mechanisms of induction and repression, Plant Cell 25 (2013) 3159–3173.
[17] I. Tasiu, U. Shahid, M. Adul, P.S. Maheshwar, Secondary metabolism of pharmaceuticals in the plant in vitro cultures: strategies, approaches, and limitations to achieving higher yield, Plant Cell Tiss. Organ. Cult. 132 (2018) 239–265.
[18] F. Skoog, C.O. Miller, Chemical regulation of growth and organ formation in plant tissue cultures in vitro, Symp. Soc. Exp. Biol. 11 (1957) 118–131 [PubMed] [Google Scholar].
[19] A. Hoque, A. Nahar, M.A. Razvy, et al., Micropropagation of water chestnut (Trapa sp.) through local varieties of Rajshahi division, Asian J. Plant Sci 5 (3) (2006) 409–413 [Crossref], [Google Scholar].
[20] S. Yasmin, K.M. Nasiruddin, R. Begum, et al., Regeneration and establishment of potato plantlet through callus formation with BAP and NAA, Asian J. Plant Sci 2 (12) (2003) 936–940 [Crossref], [Google Scholar].
[21] F. Shirin, M. Hossain, M.F. Kabir, et al., Callus induction and plant regeneration from internodal and leaf explants of potato (Solanum tuberosum L.) cultivars, World J. Agric. Sci. 3 (2007) 16.
[22] A.M.J. Aljibouri, K.W. Al-samarraei, A.S. Abd, D.M. Mageed, A.A. Ali, Alkaloids Production from Callus of Hyoscyamus niger L .in Vitro, J. Life Sci. 6 (2012) 874–882.

[23] A.I. Ibrahim, M. Abd El Kawi, A. Nower, A. Abdel Motaal, A. Abdel Aal, Alkaloids production and organogenesis from callus of Hyoscyamus muticus L. In vitro, J. Appl. Sci. Res. 5 (2009) 382–392.
[24] G. Fu, Z. Haidao, L. Wu, Hu. Xingming, H. Ning, Q. Qian, X. Lin, B. Hongwu, Callus initiation from root explants employs different strategies in rice and arabidopsis, Plant Cell Physiol. 59 (9) (2018) 1782–1789.
[25] R. Abd-Rahman, E.H. El-Din, A. El-Said, H.D. Khlifa, Production of scopolamine and hyoscyamine in callus and regenerate cultures of Datura metel (L.), J. Appl. Sci. Res. 4 (2008) 1858–1866.
[26] M.K. Mutasim, G.A.E. Khadiga, S.M. Rasheid, Callus formation and organogenesisof potato (Solanum tuberosum L.) cultivaralmera, J. Phytology 2 (5) (2010) 40–46.
[27] A. Nistor, G.H. Campeanu, C. Nicoleta, K.C. Diana, Effect of auxin and cytokinin on callus induction in potato (Solanum tuberosum L.) explants, Agricultura Stiintasipractica 12 (69–70) (2009) 47–50.
[28] S.R. Adhikari, B. Pant, Induction and Proliferation of in vitro Mass of Callus of Withania somnifera L. Dunal, Research in Plant Sciences 1 (3) (2013) 58–61.
[29] Beem-Usa Inwanna Van, Sainiya Samala, Chanpradit Charuvat, Choosingh Natthaya, Effects of Culture Media and Explant Types on Callus and Shoot Induction of Annona muricataL, Malaya J. Biosci. 1 (3) (2014) 155–159. ISSN 2348-6236 print /2348-3075 online.
[30] B. Suraiya, Mostafiz, W Alina, Efficient callus induction and regeneration in selected Ind, Agronomy 8 (2018) 77.