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Research article

Callus Formation and Regeneration Tendency of *Justicia vasica* Nees under Saline Conditions

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Abstract

Salinity is one of the major environmental threats to plants because a large number of plants are intolerant to NaCl stress. One such plant, *Justicia vasica*, which is the subject of our study, is said to have great medicinal properties. However, the natural occurrence and cultivation of this species has been constrained due to various factors including its poor seed germination rate under its normal conditions of habitat, and a low availability of seeds. This study was undertaken to examine the efficiency of callus formation and regenerative tendencies of *Justicia vasica* under saline conditions. The leaf, shoot tips and inter nodal cuttings were employed for this study using MS media formulated with several concentrations of different kinds of hormonal combinations. This study also tested the effects of different concentrations of NaCl on callus induction and growth. Each experiment was conducted thrice with 12 replicates. A superior level of callus formation was achieved by treating shoot tips with 2.0 mg/l α-naphthalene acetic acid (NAA) and 0.5 mg/l 6-benzyladenine purine (BAP) in combination. From this study, it was concluded that there was no inhibitory action from the medium supplemented with 0.05 mg/l NaCl. The optimal hormonal combination also exhibited highest rate of callus induction and growth with 0.05 mg/l NaCl added. There was no callus necrosis at this saline concentration after 60 days of inoculation. Furthermore, some somatic embryogenic callus displayed the organogenesis of leaf like structures after 75 days of inoculation. The development of salinity tolerance of plants by genetic application has been a vital area on modern agriculture as well as medicinal plant research because more land has been salinized through poor local irrigation practices. The present investigation assumed that the NaCl tolerance of this plant could be effectively obtained *in vitro*.

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1. Introduction

Justicia vasica Nees. (Acanthaceae), locally known as 'Basak' in Bengali, is an evergreen woody shrub distributed in south-east Asia [1, 2]. The plant is used to treat asthma, respiratory tract disorder, cough as well as throat infection [3, 4]. This plant contains various types of secondary metabolites such as industrially important vasicine, adhavasinone, vasnetine and vasicinone alkaloids found in every parts of the plant [1, 5]. These metabolites show restrictive activity against *Mycobacterium tuberculosis*, human immunodeficiency virus (HIV) infection and other infectious diseases [6]. Other medicinal applications of this plant are for antispasmodic, fever reducer, anti-inflammatory, anti-bleeding, anti-diabetic, anti-jaundice, antiseptic and expectorant activities [2, 7]. The leaf extracts of this species are added to drinking water or juice to treat germs and harmful insects [8]. Due to its high degree of medicinal value, this species is listed as one of the top 22 medicinal plants exported from India [9]. Furthermore, because of its significant therapeutic properties and its high consumption rate (more than 100 metric ton per year), it is scheduled under the top 36 medicinal plant species in India by NMPB (National Medicinal Plants Board), New Delhi [9]. However, the circulation of this plant species has been constrained because of low seed availability and poor seed germination rate [5]. Moreover, its cultivation through shoot cuttings for multiplication is also slow due to seasonal dependency [1]. Surprisingly, very few studies have been undertaken on *in vitro* preservation of medicinal species including this plant [8]. Scanty methodical cultivation, low propagation response, high utilization and inadequate scientific studies have resulted in rapid depletion of plant material of this species.

Salinity is among the major environmental problems and risks for agricultural crops as well as other plants in arid and semiarid regions of the world [10, 11]. Nowadays, the fertility of arable land is being harmed as levels of sodium chloride (NaCl) stress in soil grow with the passage of time. Rising salt level have reduced agricultural yields by about 20% globally as about one-third of all arable lands has turned saline [10, 12]. As a result, increased number of plants have been negatively affected by the development of saline environments. With this situation in mind, in order to meet the needs of the pharmaceutical industry as well as for other purposes, this medicinal plant needs to be cultured conventionally in saline soils. *In vitro* technique illustrated NaCl tolerant status in a short period of time and in more stable form of plant species [13]. *In vitro* study provides a powerful tool to advance salinity tolerance via somaclonal variation, which contributes to plantlet a genetic transformation. In this circumstance, it is necessary to develop a well-organized procedure for callus proliferation for *in vitro* selection of salt tolerant plant material against salinity stresses [14]. The tissue culture study may give promising results as the capacity of plants to develop under NaCl stress increased when the plants survived a steady increase in NaCl concentration in the culture medium at cellular level as callus culture [15].

Therefore, the purpose of this study was to screen for the best hormonal condition for efficient callus formation of *J. vasica* towards conservation. The second purpose was to find the highest level of salt concentration in callus induction medium that this species can tolerate and retain the ability to induce callus formation.

2. Materials and Methods

2.1. Plant material and surface sterilization

Various juvenile disease-free (leaf, shoot tip, inter nodal) explants were collected from matured plant, situated in the medicinal garden of the Department of Botany, University of Kalyani, West Bengal. After the excision, the explants were dipped into water to bring them into the laboratory.

Thereafter, the explants were cleaned thoroughly in running tap water, washed with 5% (v/v) teepol for 15 min, and then rinsed three times in sterile distilled water. Afterwards the explants were surface disinfected with 70% alcohol for 1 min followed by treatment with 0.1% (w/v) $HgCl_2$ solution for 10 min and rinsing with sterile distilled water three times under a laminar air flow. The processed explants were then sized to 1-1.2 cm for inoculation on culture media.

2.2. Culture media and conditions

The sterilized explants (1.2 cm long) were cultivated on Murashige and Skoog (MS) medium [16] bearing 3% (w/v) sucrose. The medium pH was kept at 5.7 prior to addition of 0.8% (w/v) agar and then sterilized with autoclaving. After inoculation, the culture tubes were kept at 24±2°C with 60%-70% relative humidity.

2.3. Prevention of browning problem of explant in culture media

After inoculation, the cultures were kept in darkness for different periods of time (2, 4, 6, 8 days) continuously to reduce phenol excretion. After the dark treatment, the whole setup was placed under fluorescent light (16 h photo periods).

2.4. Effect of hormonal combinations and concentrations

For this study, two auxins at different concentrations (0.5, 1, 1.5, 2, 2.5 and 3 mg/l) were observed viz, 2, 4-dichloro phenoxy acetic acid (2, 4-D) and α-naphthalene acetic acid (NAA) in combination with 6-benzyladenine purine (BAP) at the concentration of 0.5 mg/l. The callus initiation rate (CIR) and callus growth (CG) were determined after 1 month and 2 months of inoculation, respectively. They were calculated with the following formulas:

$$\text{CIR} = \frac{Number\ of\ exlants\ having\ callus\ in\ \ medium}{Total\ number\ of\ explants\ planted\ for\ callus\ in\ the\ same\ medium} \times 100$$

CG = Final weight of callus - initial inoculation weight

2.5. Effect of salt concentrations in the medium

The salt tolerant was determined by adding NaCl in the medium at various concentrations such as 0.05, 0.1, 0.15, 0.20, 0.25, 0.30 mg/l. The cultures were maintained with constant subculturing at 15-day intervals on freshly prepared MS medium formulated with optimized hormonal conditions.

2.6. Data collection and statistical analysis

Each experiment was conducted thrice with 12 replicates. The experimental data were processed by one way analysis of variance (ANOVA) and the variation between means were calculated using Duncan's Multiple Range Test $P \leq 0.05$ [17] on the statistical package of SPSS (Version 10).

3. Results and Discussions

3.1. Selection of explants for callus initiation

Among the different explants considered for this investigation, leaves and inter-nodal segments proved to be unsuitable for callus induction (Figures 1a and 1b). Callus was achieved from the shoot tips (Figures 1c and 1d). After this observation each experiment of this study was conducted with 1.2 cm long shoot tips.

The selection of perfect explant is the key to establish a successful *in vitro* study. The success depends on various factors like age, size, position, collection time of explant, etc. However,

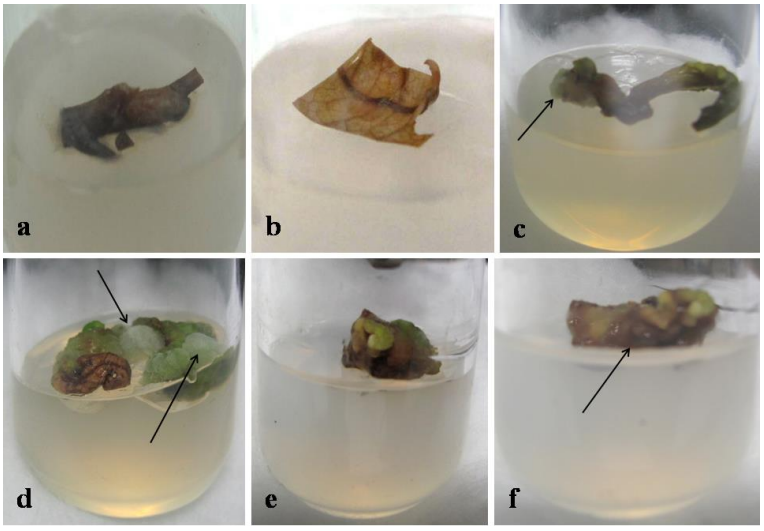

Figure 1. Callus formation and growth under several concentrations of various plant hormones and NaCl, a. Browning of inter-nodal segment in medium showing no formation of callus, b. Leaf disc in medium showing browning of explant and no callus formation, c. Initiation of callus at 2.0 mg/l NAA and 0.5 mg/l BAP after 8 days of incubation indicating callus initiation site with black arrow, d. Callus after 30 days of inoculation indicating formation of globular structure in the callus by black arrows at 2.0 mg/l NAA and 0.5 mg/l BAP in medium, e. Development of brown callus indicated with black arrow on 2, 4-D (3.0 mg/l) and BAP (0.5 mg/l) after 30 days of incubation, and f. Callus on 0.20 mg/l NaCl containing medium after 30 days of incubation

the most important factor is the occurrence of meristematic tissues in explant. In this study, the shoot tip gave suitable results for callusing of this species. Similar reports with this species were also made by other researchers [18, 19]. The shoot tip culture has shown many advantages including better response and virus free results over other explants developed earlier [20]. Moreover, shoot tip usually contains high level of IAA, which helps to create a perfect environment for callus induction and successive organogenesis experiments, and offers a promising clarification for the bountiful callus formation examined in this study [21].

3.2. Prevention of browning problem

Explant browning was eliminated by keeping the cultures in the dark continuously for 8 days and the process showed considerable result. The browning of explants (Figures 1a and 1b) during tissue culture in culture medium is one of the vital constraints for many plant species, especially for woody species. Usually explant browning is affected by emission of phenolic constituents from the cut ends of explants and its callus restriction mechanism was observed by Al-Mayahi *et al*. [22]. This colorization of explants can be controlled by the addition of phenol absorbents, antioxidants and chelating agents into the medium or by transferring the explants into fresh culture media at regular times according to several reports [23, 24]. However, observation based on this investigation seemed that additional compounds may increase the media complexity which may further interfere with callus induction, and in sequential methodologies the procedure becomes tedious and time consuming.

3.3. Results of 2, 4-D and NAA in association with BAP on callus formation

Callus initiation was examined on MS medium formulated with various concentrations of 2, 4-D in association with BAP. Callus was induced from the sliced section of explants after 12 days of inoculation, primarily sprouting of shoot tips was observed. At lower concentrations (0.5-1.5 mg/l) of 2, 4-D with BAP (0.5 mg/l), white yellow callus was observed whereas at higher levels (2-3 mg/l) of 2, 4-D light brown callus was formed (Table 1 and Figure 1e). This hormonal combination induced comparatively lower amounts of callus and the calli that formed were fragile in texture (Table 1). As the concentration of 2, 4-D was increased (1.5-3.0 mg/l), the fragility and brownness increased simultaneously. The rate of growth of callus was also slow and the growth was retarded after 6 weeks. The callus turned brown and could not be used for further studies.

This may happen because the synthetic hormone 2, 4-D affects the morphological abnormalities of callus by causing physiological disorders or somaclonal variation [25]. Moreover, a recent study found that it has harmful effect on plants, i.e. it can change the chlorophyll, protein and phenol content [26]. The browning of callus displays the sensitivity of plant cells to 2, 4-D. The browning is mainly caused by the action of phenolic compounds present in cells. When these compounds accumulate in cytoplasm, they undergo oxidation and polymerization and the oxidized materials are responsible for the visible brown colour [27, 28].

The callus formation was also tested on MS medium containing several concentrations of NAA in combination with BAP. Callus was initiated after 8 days of incubation at both cut ends of inoculums. The combination of BAP and NAA produced yellow and dark green callus (Figures 1c and 1d). The best callus formation (callus induction rate of explants) and growth (enlargement of callus) was exhibited with 2.0 mg/l NAA and 0.5 mg/l BAP in combination. At lower amount of NAA (0.5-1.0 mg/l) with 0.5 mg/l BAP, a lower quantity of white callus was initiated as compared to the higher quantities of NAA with 0.5 mg/l BAP (Table 1). Among the various associations of phytohormones examined, the NAA and BAP was found to be superior to other combinations as it produced elevated frequency of embryogenic greenish dense callus. This treatment (higher quantities of NAA with 0.5 mg/l BAP) also displayed a number of greenish somatic embryogenic calli. In NAA supplemented medium, the callus showed fast growth and for this reason 2.0 mg/l NAA and 0.5 mg/l BAP combination medium was selected for detection of NaCl tolerance.

The present study examined only one cytokinin at 0.5 mg/l concentration which was previously reported by various workers to be effective for callus formation [29]. A small quantity of BAP had a tremendous role in callusing by promoting RNA and protein synthesis, which were required for the formation of various enzymes involved in cell wall loosening and cell cycling activity [30]. Among the different growth regulators, 2, 4-D is preferable for callus formation [31,

32]. In this study, however, NAA gave more significant results than 2, 4-D when used along with 0.5 mg/l BAP. Similar types of findings were also reported by several authors globally with different types of plant species [28, 33].

3.5. Effect of NaCl on callus induction, development and callus necrosis

The main objective of this experiment was to see the effect of different salt concentrations on callus induction and growth. The action of NaCl on callusing is shown on Table 2. From this study, it was concluded that there was no inhibitory action of salt added to the medium at 0.05 mg/l NaCl, but when the concentration of salt was increased as per experimental design, the callus response and growth of the callus were delayed (visually) and the produced calli were slightly brown in colour (Figures 1f and 2a).

Table 1. The callus response, texture and colour under different concentrations of various growth regulators in MS medium

Growth Regulators (mg/l)			% of Callus Initiation Rate (CIR)	Texture of Callus	Colour of Callus
2, 4-D	NAA	BAP			
00	00	00	0000±0.00^{f}	-	-
0.5	-	0.5	30.76±0.04^{e}	Compact	White
1.0	-	0.5	46.15±0.04^{cde}	Compact	White
1.5	-	0.5	64.10±0.02^{abcd}	Fragile	White yellow
2.0	-	0.5	66.66±0.02^{abc}	Fragile	Light brown
2.5	-	0.5	76.92±0.04^{a}	Fragile	Light brown
3.0	-	0.5	71.79±0.02^{ab}	Fragile	Brown
-	0.5	0.5	51.28±0.02^{bcd}	Compact	White
-	1.0	0.5	51.38±0.02^{bcd}	Compact	White yellow
-	1.5	0.5	44.76±0.19^{de}	Compact	White greenish
-	2.0	0.5	84.61±0.04^{a}	Compact	Greenish
-	2.5	0.5	74.36±0.05^{a}	Compact	Greenish
-	3.0	0.5	69.23±0.04^{ab}	Compact	Yellow

Note: The values in the second column are Mean ± SE followed by the letters within the column indicating the level of significance at $P \leq 0.05$ by Duncan's Multiple Range Test.

Table 2. Callus growth and necrosis under different concentrations of NaCl

Concentration of NaCl (mg/l)	% of Callus Initiation Rate (CIR)	Callus Growth (CG)	Callus Necrosis (Days after Initiation)
0	84.61±0.04^{a}	++++	No necrosis
0.05	82.05±0.02^{a}	++++	No necrosis
0.10	71.79±0.02^{a}	+++	30
0.15	56.40±0.05^{b}	+	15
0.20	46.15±0.04^{b}	+	8
0.25	30.76±0.04^{c}	-	-
0.30	07.33±0.04^{d}	-	-

++++ Indicates fast growth, +++ Indicates slightly growth inhibition, + poor callus growth, - no growth. The values in the second column are Mean ± SE followed by the letters within the column indicating the level of significance at $P \leq 0.05$ by Duncan's Multiple Range Test.

Callus necrosis by NaCl has previously been reported by a number of researchers. In this study, the effect of NaCl on callus necrosis is displayed in Table 2. The study clearly indicated that callus necrosis started at 0.10 mg/l NaCl containing medium after 30 days of callus initiation whereas 0.05 mg/l containing medium showed no necrosis after 60 days of callus initiation (Figure 2b). Furthermore, after 75 days of inoculation there was formation of leaf like structures in callus (Figure 2c). Finally, media with NaCl of 0.20 mg/l or above showed poor growth and callus necrosis at 8 days from the date of callus initiation.

There is not always a strict relationship between salt-tolerance of whole plants and cultured cells *in vitro*. NaCl causes metabolic dysfunction by ion toxicity, osmotic stress and nutritional deficiency [13, 34]. Callus necrosis seen with increased NaCl concentration *in vitro* may be caused

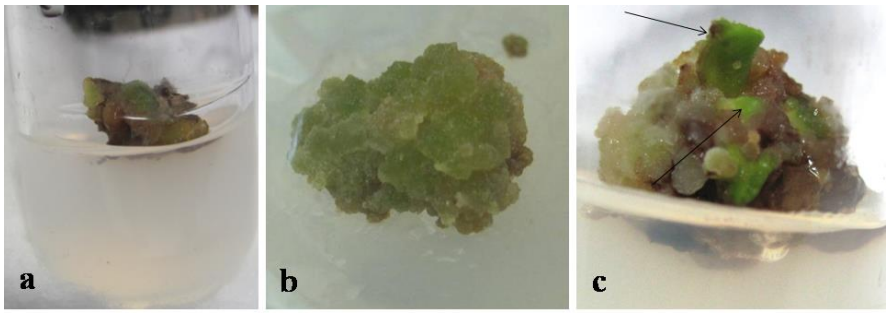

Figure 2. Callus formation and growth under several concentrations of various plant hormones and NaCl, a. Callus on 0.20 mg/l NaCl containing medium after 60 days of interval from inoculation, b. Formation of embryogenic callus on 0.05 mg/l NaCl containing medium after 51 days of inoculation with 2.0 mg/l NAA and 0.5 mg/l BAP in medium, c. Proliferation of callus on 0.05 mg/l NaCl containing medium after 75 days of inoculation showing formation of leaf with black arrows

by accumulation of several correlative compounds, for example, free amino acids (proline, betaine etc), sugars and polyphenols. Such compounds play a vital function in compensating for created osmotic stress due to the presence of NaCl in plants [35, 36]. Their biosynthesis is activated in response to applied stress. These biosynthetic products can control the uptake and placement of salts. In this way, these compounds inhibit the toxic effects of salt on the photosynthesizing apparatus where metabolic processes are going on. Furthermore, it was found that in order to neutralize increase concentrations of NaCl, salt-resistant callus lines generated higher enzymatic activities of several enzymes like ascorbate, superoxide dismutase and glutathione reductase than salt-sensitive parental cells [37]. Callus is being widely accepted in afforestation programmes [38]. So, the current study supports the preservation of this species *in vitro*. Callus culture also gives an option for genetic cell transformation study by induced mutagenesis and somaclonal modifications [39]. The development of salinity tolerance of plants by genetic application has been a vital area of modern agriculture as well as medicinal plant research because more land has become salinized through poor local irrigation practices. To solve this problem a rapid advancement in understanding of biochemical pathways that participate in salt tolerance and plant responses including genetic engineering of genes concerned in different metabolic mechanisms that counter NaCl stress is

needed worldwide [40]. It is an established fact that a complete degree of salt tolerant (*in vitro*), in terms of nutrient compositions, phytohormones, temperature and humidity level, is needed for each explant of plant species. The effect of concentration and type of auxin and cytokinin were very important not only on the percentage of callus production, but also on callus growth and cell proliferation.

4. Conclusions

In this study, the effects of different combinations of auxins and cytokinins on callus induction were studied with different explants. The greatest number of callus formation was achieved by treating shoot tips with 2.0 mg/l NAA and 0.5 mg/l BAP in combination. From this study, it was cleared that there was no inhibitory action when the medium was supplemented with 0.05 mg/l NaCl. The present experiment revealed that the shoot tips of *J. vasica* reacted to various NaCl concentrations and their effectiveness was assessed in *in vitro* culture. It was assumed that NaCl tolerance at the concentration of 0.05 mg/l for this plant could be effectively obtained *in vitro* as a hopeful alternative for traditional field assessments.

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References

[1] Khan, S., Akter, S., Habib, A., Banu, T.A., Islam, M., Khan, N.F., Afrin, S., Ferdousi, A. and Islam, S., 2016. Establishment of *in vitro* regeneration protocol for *Adhatoda vasica* Nees. *Bangladesh Journal of Scientific and Industrial Research*, 51(1), 75-80.

[2] Chakrabarty, A. and Brantner, A.H., 2001. Study of alkaloids from *Adhatoda vasica* Nees on their anti-inflammatory activity. *Phytotherapy Research*, 15, 532-534.

[3] Shinwari, Z.K., Khan, I., Naz, S. and Hussain, A., 2009. Assessment of antibacterial activity of three plants used in Pakistan to cure respiratory diseases. *African Journal of Biotechnology*, 8(24), 7082-7086.

[4] Bimal, R. and Shahnawaz, M.D., 2012. Plant regeneration from nodal explants of *Adhatoda vasica* Nees. *Journal of Medicinal Plants Research*, 6(7), 1229-1233.

[5] Prajila, P. and Indulekha, P., 2016. Shoot organogenesis in *Justicia adhatoda* (L). Nees. *International Journal of Botany Studies*, 1(7), 21-22.

[6] Grange, J.M. and Snell, N.J., 1996. Activity of bromehexine and ambroxol, semisynthetic derivatives of vasicine from the Indian shrub *Adhatoda vasica* against *Mycobacterium tuberculosis in vitro*. *Journal of Ethnopharmacology*, 50(1), 49-53.

[7] Maurya, S. and Singh, D., 2010. *In vitro* callus culture of *Adhatoda vasica*: A medicinal plant. *Annals of Biological Research*, 1(4), 57-60.

[8] Anand, Y. and Bansal, Y.K., 2002. Synthetic seeds: a novel approach of *in vitro* plantlet formation in vasaka (*Adhatoda vasica* Nees.). *Plant Biotechnology*, 19(3), 159-162.

[9] Lone, S.A., Wani, S.H. and Tafazul, M., 2017. Cost effective protocol for *in vitro* propagation of *Adhatoda vasica* Nees. using modified tissue culture media. *International Journal of Advanced Science and Research*, 2(2), 67-69.
[10] Dagar, J.C., 2005. Ecology, management and utilization of halophytes. *Bulletin of the NIE*, 15, 81-97.
[11] Khenifi, M.L., Boudjeniba, M. and Kameli, A., 2011. Effects of salt stress on micropropagation of potato (*Solanum tuberosum* L.). *African Journal of Biotechnology*, 10(40), 7840-7845.
[12] Atabaki, N., Nulit, R., Kalhori, N., Lasumin, N., Sahebi, M. and Abiri, R., 2018. *In vitro* selection and development of Malaysian salt-tolerant rice (*Oryza sativa* L. cv. MR263) under salinity. *Acta Scientific Agriculture*, 2(8), 8-17.
[13] Saputro, T.B., Dianawati, S., Sholihah, N.F. and Ermavitalini, D., 2017. Genetic diversity of improved salt tolerant calli of maize (*Zea mays* L.) using RAPD. *AIP Conference Proceedings*, 1854, 020033, https://doi.org/10.1063/1.4985424.
[14] Benderradji, L., Brini, F., Kellou, K., Ykhlef, N., Djekoun, A., Masmoudi, K. and Bouzerzour, H., 2012. Callus induction, proliferation, and plantlets regeneration of two bread wheat (*Triticum aestivum* L.) genotypes under saline and heat stress conditions. *ISRN Agronomy*, 2012, https://doi.org/10.5402/2012/367851.
[15] Nasser, S., Iwan, D.T. and Paul, J.K., 1980. Effect of repeated application of water stress on water status and growth of wheat. *Physiologia Plantarum*, 50(1), 11-15.
[16] Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), 473-497.
[17] Duncan, D.B., 1955. Multiple Range and Multiple F Test. *Biometrics*, 11(1), 1-42.
[18] Khalekuzzaman, M., Rahman, M.S., Rashid, M.H. and Hossain, M.S., 2008. High frequency in vitro propagation of *Adhatoda vasica* Nees through shoot tip and nodal explants culture. *Journal of Bio-Science*, 16, 35-39.
[19] Tejavathi, D.H., Manjula, B.L. and Anitha, P., 2010. Multiple Shoot Regeneration from the Cultures of *Adhatoda vasica* Nees. *Acta horticulturae*, 865, 367-370.
[20] Adesoye, A.I., Okooboh, G.O., Akande, S.R., Balogun, M.O. and Odu, B.O., 2012. Effect of phytohormones and genotype on meristem and shoot tip culture of *Telfairia occidentalis* Hook F. *Journal of Applied Biosciences*, 49, 3415- 3424.
[21] Isah, T., 2019. De novo *in vitro* shoot morphogenesis from shoot tip-induced callus cultures of *Gymnema sylvestre* (Retz.) R.Br. ex Sm. *Biological Research*, 52, 3, https://doi.org/ 10.1186/ s40659-019-0211-1.
[22] Al-Mayahi, A.M.W., Ali, A.H. and Shareef, H.J., 2018. Influence of cold pretreatment on shoot regeneration from callus in date palm (*Phoenix dactylifera* L.) cv. 'Barhee'. *Journal of Genetic Engineering and Biotechnology*, 16(2), 607-612.
[23] Altan, F., Bürün, B. and Sahin, N., 2010. Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization. *African Journal of Biotechnology*, 9(7), 991-995.
[24] Singh, C.R., 2018. Review on problems and its remedy in plant tissue culture. *Asian Journal of Biological Sciences*, 11(4), 165-172.
[25] Garcia, C., Almeida, A.F.D., Costa, M., Britto, D., Valle, R., Royaert, S. and Marelli, J.P., 2019. Abnormalities in somatic embryogenesis caused by 2, 4-D: an overview. *Plant Cell, Tissue and Organ Culture*, 137, 193-212.
[26] Peixoto, F.P., Gomes-Laranjo, J., Vicente, J.A. and Madeira, V.M.C., 2007. Comparative effects of the herbicides dicamba, 2,4-D and paraquat on non-green potato tuber calli. *Journal of Plant Physiology*, 165, 1125-1133.
[27] Lukas, A.M., Christoper, D.G., Rebecca, A.S. and Virgnia, W., 2000. AN9-a petunia glutathione s-transferase required for anthocyanin sequestration, is a flavonoid binding protein. *Plant Physiology*, 123, 1561-1570.

[28] Sridhar, T.M. and Naidu, C.V., 2011. An efficient callus induction and plant regeneration of *Solanum nigrum* (L.) - An important antiulcer medicinal plant. *Journal of Phytology*, 3(5), 23-28.
[29] Seyyedyousefi, S.R, Kaviani, B., Dehkaei, N.P. and Salehzadeh, A., 2013. Callus induction in Alstroemeria using NAA and BAP. *European Journal of Experimental Biology*, 3(5), 137-140.
[30] Khan, N., Ahmed, M., Hafiz, I., Abbasi, N., Ejaz, S. and Anjum, M., 2015. Optimizing the concentrations of plant growth regulators for *in vitro* shoot cultures, callus induction and shoot regeneration from calluses of grapes. *Journal International des Sciences de la Vigne et du Vin*, 49, 37-45.
[31] Michel, Z., Hilaire, K.T., Mongomaké, K., Georges, A.N. and Justin, K.Y., 2008. Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). *Australian Journal of Crop Science*, 2(1), 1-9.
[32] Sundarasekar, J., Anthony, J.J.J., Murugaiyah, V. and Subramaniam, V., 2012. Preliminary responses of 2, 4-D and BAP on callus initiation of an important medicinal-ornamental *Hymenocallis littoralis* plants. *Journal of Medicinal Plants Research*, 6(11), 2088-2093.
[33] Ahmad, S. and Spoor, W., 1999. Effects of NAA and BAP on callus culture and plant regeneration in Curly Kale (*Brassica oleraces* L.). *Pakistan Journal of Biological Sciences*, 2 (1), 109-112.
[34] Rahman, M.H., Islam, R., Hossain, M. and Haider, S.A., 2009. Differential response of potato under sodium chloride stress conditions *in vitro*. *Journal of Bio-Science*, 16, 79-83.
[35] Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J., 2000. Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology*, 51, 463-499.
[36] Ashraf, M. and Foolad. M.R., 2007. Roles of glycinebetaine and proline in improving plant abiotic stress resistance. *Environmental and Experimental Botany*, 59(2), 206-216.
[37] Efferth, T., 2019. Biotechnology applications of plant callus cultures. *Engineering*, 5, 50-59.
[38] Ahuja, M.R., 1991. Application of biotechnology to preservation of forest tree germplasm. In: M.R. Ahuja, ed. *Woody Plant Biotechnology*. New York: Plenum Press, pp. 307-313.
[39] Ahmad, N., Faisal, M., Anis, M. and Aref, I.M., 2010. *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. *South African Journal of Botany*, 76(3), 597-600.
[40] Winicov, I. and Bastola, D.R., 1997. Salt tolerance in crop plants: new approaches through tissue culture and gene regulation. *Acta Physiologiae Plantarum*, 19, 435-449.