ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 3, pp: (123-129), Month: July - September 2019, Available at: www.researchpublish.com

Micropropagation of *Asparagus officinalis* L. (Garden Asparagus) *In Vitro*

Rasad, F. M.[1*], Hasbullah, N. A.[1], Azis, N. A.[1], Daud, N. F.[1] and Lassim, M.M[2]

[1] Department of Agricultural Science, Faculty of Technical and Vocational, Universiti Pendidikan Sultan Idris (UPSI), Tanjung Malim, Perak, Malaysia

[2] Faculty of Sustainable Agriculture (Sandakan Campus), Universiti Malaysia Sabah (UMS), 90000, Sandakan, Sabah, Malaysia.

Email: caeq_pex@yahoo.com

Abstract: ***Asparagus officinalis*** **L. is a herbaceous plant belongs to liliaceae family with semi-erect and branched articulated branches and thick and white root, between 30 and 100 cm in length and 0.7 to 1 cm in thickness and dioecious perennial herb and is native to the Asia, Africa and Europe. This study highlights micropropagation of** ***Asparagus officinalis*** **L. through tissue culture techniques.** ***Asparagus officinalis*** **L. seeds were first washed throughly under running tap water for two hour. And then seed were soaked with distilled water for 24 hours. Seeds were then rinsed using 70%, 50%, 20% and 10% sodium hypochlorite followed by distilled water. This series of seed sterilization was done sequentially. Subsequently seeds were rinsed with 70% ethanol and finally with distilled water prior to culture. Seeds were cultured on Murashige and Skoog, 1962 (MS) basal medium containing 8% agar technical and 30% sucrose.** ***In vitro*** **seed germination was observed within two weeks. Complete plantlets were obtained after four weeks of culture. Explants such as stem and root will later be transferred onto MS medium supplemented with various concentrations of plant growth regulator such as Benzylaminopurine (BAP) and Napthalene Acetic Acid (NAA) to see further organogenesis response. The study showed that in vitro propagation of** ***Asparagus officinalis*** **L. to develop new plantlets was successfully obtained.**

Keywords: ***Asparagus officinalis*** **L., micropropagation,** ***in vitro*****, plantlets, plant growth regulators.**

I. INTRODUCTION

Asparagus is a large genus comprising more than 150 different species of high economic value perennials crop with 2n = 20 as its chromosome number. It is grown worldwide and basically originated from Asia, Africa, and Europe [1]. Asparagus is the main genus of the *liliaceae* family [2], and it is the most economically important which is a highly priced vegetable [3]. Asparagus is a popular vegetable because it is one of the first field crops to be harvested in spring and it can provide both growers and consumers with an early season fresh commodity. As a dioecious crop, asparagus is inevitably cross - pollinating. Male and female flowers are born on different plants. Generally, male plants have more commercial advantages over the female plants. They have higher productivity [4] and produce more stalks [5].

The wild stock of this high value plant species has been rapidly diminished due to over exploitation and no efforts for its replenishment has been undertaken till date. The conventional method of propagation of this plant is through seeds. However, propagation of *Asparagus officinalis* L. through seeds is unreliable due to poor rate of seed germination and a slim chance to survive under natural conditions. The conventional method is not attractive approach for the production of abundant elite plants within a short period of time. In order to meet the extensive demand of this edible plant which also has medicine properties, the alternative method to be employed in conserving its diminishing population is *in vitro* culture. *In vitro* techniques are considered as easy and reliable methods for the rapid propagation of plants, especially medicinally important plants [6] [7]. Large number of aseptic plants could be produced within short time using plant tissue culture protocols.

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 3, pp: (123-129), Month: July - September 2019, Available at: www.researchpublish.com

In fact, plant tissue culture is an alternative method for commercial propagation of abundant plant species that also include plants with medicine properties [8]. In recent years, *in vitro* culture techniques have been increasingly receiving interests because they not only offer a viable tool for mass multiplication, but they also offer germplasm conservation for rare medicinal plants that are endangered and threatened [9], [10], [11], [12]. The *in vitro* technique increases the availability of disease free stock plants and reduces culture space requirement as well as lowers the cost of production.

II. MATERIAL AND METHOD

The seed of *Asparagus officinalis* L. (Garden asparagus) were obtained from Vaxer company in Malaysia. Seeds of *Asparagus officinalis* L. were washed throughly under running tap water for two hour. And then seed were soaked with distilled water for 24 hours in the plant science laboratory. Seeds were then surface sterilized with different concentrations of sodium hypochlorite, 70% and 50% for 10 minutes and followed by 20% and 10% for 5 minutes. After that, seed were rinsed throughly with distilled water for 5 minutes. Finally, the seeds were dipped in 70% ethanol for 1 minute and rinsed with sterile distilled water all over again in laminar flow cabinet. And then, they were dried on a sterile filter paper. Seeds were germinated aseptically on MS (Murashige and Skoog, 1962) [13] basal medium supplemented with 8% agar technical and 30% sucrose. This medium was used throughout this study as the basal medium. Culture were incubated in dark photoperiod at 26°C .Seeds were germinated after 14 days of culture. Prior to germinating, cultures were transferred to cool-white-fluorescent light room and incubated at 25 ± 1°C with 16 hours light and 8 hours dark photoperiod.

Stem and root explants excised from *in vitro* plantlets containing were then placed on MS medium containing 30 g/L sucrose supplemented with various concentrations of BAP (0.5 - 2.0 mg/L) and NAA (0.5 – 2. 0 mg/L) (Tables I)(Table II). Media was solidified with 8 gram agar and autoclaved at 121°C at psi 21 minutes. Ph of media was adjusted to 5.8 prior to autoclaving. All cultures at 25 ± 1°C under white fluorescent light of 40-60 µ mol m-2 s-1 intensity for 16 hrs light /8 hrs dark photoperiod were incubated. Total number of explants in each treatment was 30. Subculturing to a new fresh medium was done after 4 weeks of cultures.

III. STATISTICAL ANALYSIS

Each treatment consists of 30 samples and each culture tube contained three explants. Experiments were repeated twice. Visual observations of the cultures were made every week and data related to frequency of organogenesis (regeneration frequency, shoots induction and root induction). The program used was SPSS version 23.0. Data were analysed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p = 0.05.

IV. RESULT AND DISCUSSION

TABLE I: The Effect of Different Concentrations and Combinations of BAP and NAA on Stem and Root Explants Cultured on MS Medium for Regeneration of Shoot at 25±1 °C with 16 Hours Light and Eight Hours Dark.

No	Treatments	Explants (Mean ± SE)	
		Stem	Root
1	0.5 mg/L BAP + 0.5 mg/L NAA	$1.900 \pm 0.194_{e}$	NR
2	1.0 mg/L BAP + 0.5 mg/L NAA	$1.900 \pm 0.154_{e}$	NR
3	1.5 mg/L BAP + 0.5 mg/L NAA	$2.300 \pm 0.263_{e}$	NR
4	2.0 mg/L BAP + 0.5 mg/L NAA	$2.733 \pm 0.318_{de}$	$4.367 \pm 0.367_{a}$
5	0.5 mg/L BAP + 1.0 mg/L NAA	$3.733 \pm 0.258_{cd}$	$3.033 \pm 0.251_{b}$
6	1.0 mg/L BAP + 1.0 mg/L NAA	$3.600 \pm 0.411_{cd}$	NR
7	1.5 mg/L BAP + 1.0 mg/L NAA	$4.600 \pm 0.459_{c}$	NR
8	2.0 mg/L BAP + 1.0 mg/L NAA	$6.233 \pm 0.810_{a}$	$4.967 \pm 0.621_{a}$
9	0.5 mg/L BAP + 1.5 mg/L NAA	$5.266 \pm 0.465_{b}$	$2.500 \pm 0.929_{b}$
10	1.0 mg/L BAP + 1.5 mg/L NAA	$3.767 \pm 0.504_{cd}$	NR
11	1.5 mg/L BAP + 1.5 mg/L NAA	$2.033 \pm 0.195_{e}$	$0.500 \pm 0.929_{de}$
12	2.0 mg/L BAP + 1.5 mg/L NAA	$1.867 \pm 0.150_{e}$	$1.800 \pm 0.388_{c}$
13	0.5 mg/L BAP + 2.0 mg/L NAA	$1.833 \pm 0.160_{e}$	$1.067 \pm 0.253_{d}$
14	1.0 mg/L BAP + 2.0 mg/L NAA	$2.267 \pm 0.203_{e}$	NR

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 3, pp: (123-129), Month: July - September 2019, Available at: www.researchpublish.com

15	1.5 mg/L BAP + 2.0 mg/L NAA	$2.033 \pm 0.162_e$	NR
16	2.0 mg/L BAP + 2.0 mg/L NAA	$1.900 \pm 0.175_e$	NR
17	0.0 mg/L BAP + 0.0 mg/L NAA	$2.267 \pm 0.291_e$	$2.567 \pm 0.324_b$

*NR: Non Responsive

In this study, explants managed to rejuvenate shoots directly and indirectly when it was cultured in culture media. Direct organogenesis occured when explants developed shoot and root after a few weeks cultured onto MS media supplemented with various combinations and concentrations of hormones. In the meantime, indirect organogenesis occured when *Asparagus officinalis* L. explants primarily formed callus and consequently the callus cells were differentiated to develop shoots. Tables I and II illustrated the response of stem and root explants cultured on MS media supplemented with different concentrations of BAP and NAA, respectively. The main purposes of this study was to determine which explants and hormone concentrations are the optimum in regenerating shoots. Therefore, evaluation was made derived from which explants and hormone concentration that generated the highest of shoot regeneration in the shortest period of time. Observations were made on the number of shoots and roots regeneration and also callus formation.

Normally, all explant responded as early two weeks when cultured on various culture media. Base on the the table I, for stem explants, the highest shoot regeneration (6.233 ± 0.810) was obtained when explants were cultured on MS medium supplemented with 2.0 mg/L BAP + 1.0 mg/L NAA (Fig. 1a). While, the lowest response for stem explant was when explants were cultured on MS medium supplemented with 0.5 mg/L BAP + 2.0 mg/L NAA (1.833 ± 0.160)(Fig. 1b). At the same time, root explants gave response to the eight treatment only. Root explants produced the highest shoot (4.967 ± 0.621) on MS medium supplemented with 2.0 mg/L BAP + 1.0 mg/L NAA (Fig. 1c). While, the lowest response for root explant was when explant were cultured on MS medium supplemented with 0.5 mg/L BAP + 2.0 mg/L NAA (Fig. 1d). These results showed that regeneration of stem into complete plants was better compared to root explants. The number of shoot differentiating from the shoot segments decreased with the 0.5 mg/L and 2.0 mg/L concentration of NAA.

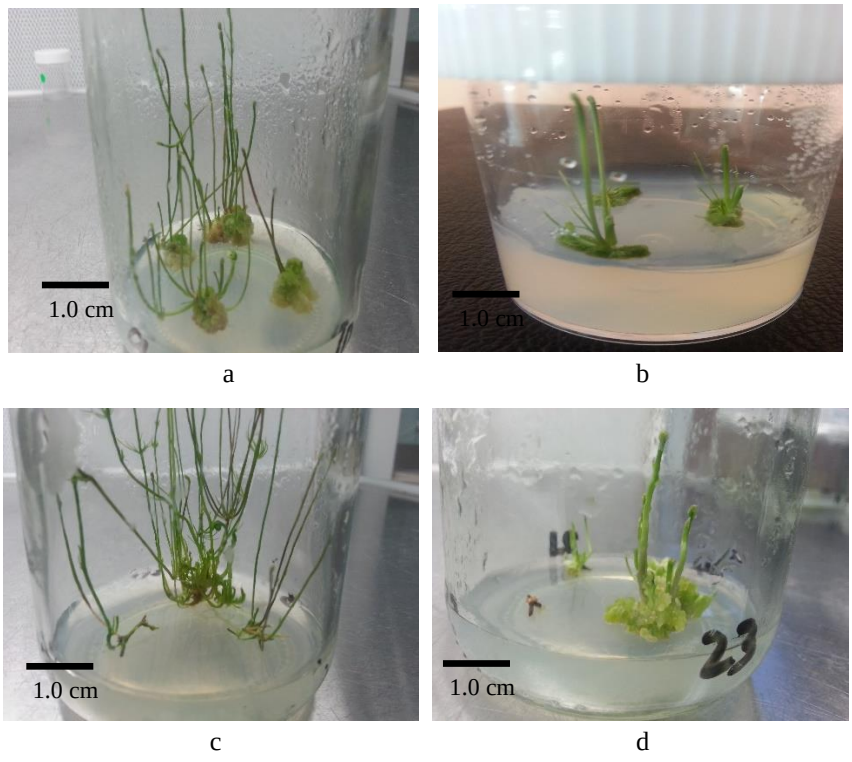


Fig. 1. Micropropagation of *Asparagus officinalis* L. (a) Shoot Regeneration from Stem Explant cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA; (b) Shoot Regeneration from Stem Explant cultured on MS medium supplemented with 0.5 mg/L BAP and 2.0 mg/L NAA; (c) Shoot Regeneration from Root Explant cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA; (d) Shoot Regeneration from Root Explant cultured on MS medium supplemented with 0.5 mg/L BAP and 2.0 mg/L NAA

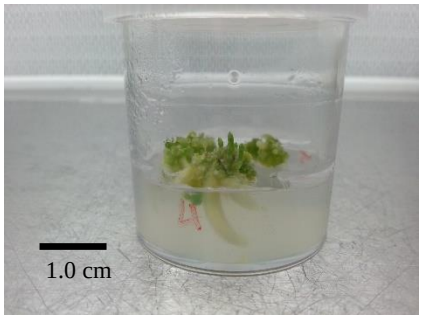
ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 3, pp: (123-129), Month: July - September 2019, Available at: www.researchpublish.com

TABLE II: The Effect of Different Concentrations and Combinations of BAP and NAA on Stem and Root Explants Cultured on MS Medium for Regeneration of Roots at 25 ± 1°C with 16 Hours Light and Eight Hours Dark.

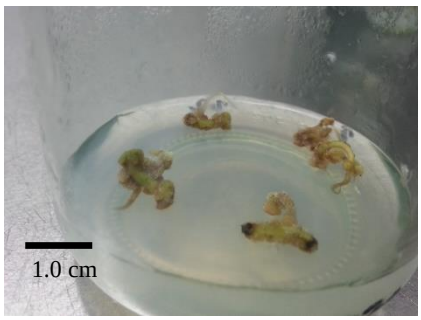
No	Treatments	Explants (Mean ± SE)	
		Stem	Root
1	0.5 mg/L BAP + 0.5 mg/L NAA	NR	$3.233 \pm 0.313_{d}$
2	1.0 mg/L BAP + 0.5 mg/L NAA	NR	$3.933 \pm 0.310_{cd}$
3	1.5 mg/L BAP + 0.5 mg/L NAA	NR	NR
4	2.0 mg/L BAP + 0.5 mg/L NAA	$3.300 \pm 0.311_{d}$	$3.700 \pm 0.468_{d}$
5	0.5 mg/L BAP + 1.0 mg/L NAA	$2.700 \pm 0.362_{d}$	$5.067 \pm 0.589_{bc}$
6	1.0 mg/L BAP + 1.0 mg/L NAA	$2.300 \pm 0.237_{de}$	$3.433 \pm 0.471_{d}$
7	1.5 mg/L BAP + 1.0 mg/L NAA	$6.767 \pm 0.894_{b}$	$8.067 \pm 0.935_{b}$
8	2.0 mg/L BAP + 1.0 mg/L NAA	$4.700 \pm 0.638_{c}$	$8.500 \pm 0.840_{a}$
9	0.5 mg/L BAP + 1.5 mg/L NAA	$10.567 \pm 0.841_{a}$	$6.000 \pm 0.670_{b}$
10	1.0 mg/L BAP + 1.5 mg/L NAA	$1.533 \pm 0.133_{e}$	NR
11	1.5 mg/L BAP + 1.5 mg/L NAA	NR	NR
12	2.0 mg/L BAP + 1.5 mg/L NAA	NR	NR
13	0.5 mg/L BAP + 2.0 mg/L NAA	NR	NR
14	1.0 mg/L BAP + 2.0 mg/L NAA	NR	NR
15	1.5 mg/L BAP + 2.0 mg/L NAA	NR	NR
16	2.0 mg/L BAP + 2.0 mg/L NAA	NR	NR
17	0.0 mg/L BAP + 0.0 mg/L NAA	NR	$3.133 \pm 0.454_{d}$

Table 4.2 described the response and development of rooting of *Asparagus officinalis* L. explant when cultured on MS media supplemented with different concentrations of BAP and NAA. From the result, only seven treatment gave response to the stem explant and nine treatment gave response to the root explant for rooting induction. Meanwhile, root explant did not respond to all treatments. For stem explants, the highest root induction (10.567 ± 0.841) was observed on MS medium supplemented with 0.5 mg/L BAP + 1.5 mg/L NAA (Fig. 2a). Meanwhile, MS medium supplemented with 1.0 mg/L BAP + 1.5 mg/L NAA was identified as the non-responsive medium for rooting of stem explant with only 1.533 ± 0.133 root per explant (Fig. 2b). Furthermore, only nine concentrations of MS medium reacted to the root explant in root induction.

MS medium supplemented with 2.0 mg/L BAP + 1.0 mg/L NAA was the best concentration for rooting of root explant with 8.500 ± 0.840 root per explant (Fig. 2c), whereas MS medium without any hormone gave lowest result for rooting of root explant with 3.133 ± 0.454 root per explant (Fig. 2d). These result showed that root induction was better achieved from stem explant. Overall, BAP hormone gave the impact in developing the growth of root for both stem and root explants. Generally, all explants successfully formed shoots on all the hormone combinations tested. Root induction was also observed from various hormone combinations. Observations was taken every week up to eight weeks of culture. Subculturing was done after the shoots have appeared and attained height about more than 20 mm. The shoots were individually separated from the plantlet clusters and transferred to initiate root induction and further shoot development. MS medium supplemented with 0.5 mg/L BAP + 1.5 mg/L NAA was selected as the most suitable medium in regeneration of *Asparagus officinalis* L.

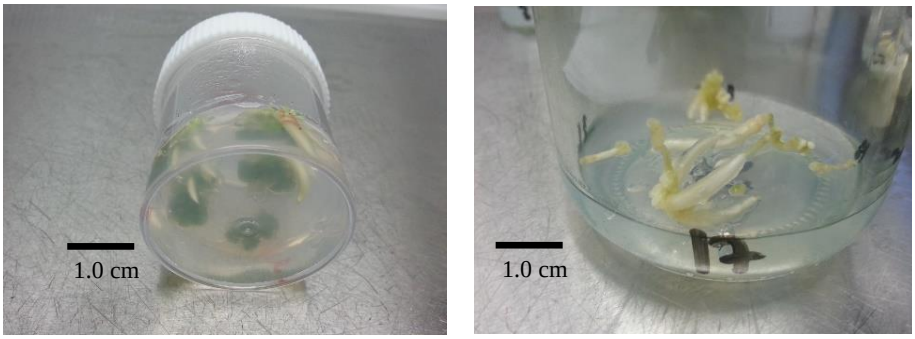


a



b

ISSN 2348-313X (Print)
International Journal of Life Sciences Research ISSN 2348-3148 (online)
Vol. 7, Issue 3, pp: (123-129), Month: July - September 2019, Available at: www.researchpublish.com



c d

Fig. 2. Micropropagation of *Asparagus officinalis* L. (a) Root Induction from Stem Explant cultured on MS medium supplemented with 0.5 mg/L BAP and 1.5 mg/L NAA; (b) Root Induction from Stem Explant cultured on MS medium supplemented with 1.0 mg/L BAP and 1.5 mg/L NAA; (c) Root Induction from Root Explant cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L NAA;(d) Root Induction from Root Explant cultured on MS medium supplemented with 0.0 mg/L BAP and 0.0 mg/L NAA

Initially, the study of *in vitro* regeneration of *Asparagus officinalis* L. was done. Many factors such as the type of explants and hormonal effects on establishment of good regeneration system for this species have been studied in order to acquire the best regeneration system for *Asparagus officinalis* L. Murashige and Skoog (MS) was used as the basic medium in this study, which was chosen based on previous studies. For instance, [14] reported that MS media supplemented with NAA alone or BAP alone were used as multiplication media for *Asparagus racemosus* Willd. Since culture medium is one of the most important components of plant cell and tissue culture methods so it is important to identify the suitable medium before further *in vitro* work can be done. Although MS medium was developed for garden asparagus (*Asparagus officinalis* L.) it has proven suitable for a variety of *liliaecae* species and is currently the most widely used medium for asparagus tissue culture [15]. It is also proven in this study that it was a suitable medium for *Asparagus officinalis* L.

Choice of explants is essential in indicating how micropropagation can be efficient [16]. Explant source is also crucial in indicating morphogenetic and regenerative possibility that is significantly determined by the donor plant's physiological conditions [17] [18]. Donor plants maintained under conditions of clean and controlled environment guarantee explants that are healthy and sterile [19]. The other factors that influence formation of organs *in vitro* also include the explants' physiological age, type, and size [20]. Table I summarizes the results obtained when stem and root explants were cultured on MS media consisting of various concentrations and combinations of BAP and NAA at 25±1ºC for 8 weeks under 16 hours light and 8 hours dark. The medium containing 2.0 mg/L BAP + 1.0 mg/L NAA showed good response on shoot regeneration. [21] has demonstrated that shoot number per explant increased with increasing BAP in *Rauwoflia serpentine*. Most studies on shoot regeneration proved that BAP played good roles in shoot and bud inductions. The studies include those done in regeneration of *Swertia ciliata, Lycopersicon esculentum* L., *Asparagus racemosus* wild and *Amomum subulatum* whereby increasing concentrations of BAP were the best to generate shoot [22], [23], [24], [25].

In the present study, root formation for stem explant was induced through the addition of 1.5 mg/l NAA. The effect of 1.5 mg/l NAA depicts the highest root formation with 10.567 ± 0.841 (Table II). Fig. 2a showed the root initiation and elongation. Addition more than 1.5 mg/L in the medium was not significant for root induction cause the explant were become no response. Thus, for root formation of this plant, the use of NAA is generally considered to be one of the most useful auxin for root formation. [24] concluded that NAA at various concentrations was effective in root formation on wild Nepalese *Asparagus racemosus*, which might be attributed to its chemical stability, low mobility in the plant and prolonged-action nature. NAA strongly excited the growth of root in *Astragalus chrysochlorus* [26]. Likewise, *Pueraria lobata* Willd Ohwi, NAA induced formation of roots in other legume [27]. It is noteworthy to address that medium containing auxin is commonly required in the process of *in vitro* root initiation, development, and elongation [28]. The success of NAA promoting efficient root induction has also been reported earlier in *Asparagus officinalis* L. [29]. Auxin is one of the major determinants for formation of root that develops to other biochemical pathways, resulting in plants' various responses [30].

V. CONCLUSION

In this study, micropropagation of Asparagus officinalis L. was successfully obtained. Stem and root explants have been identified as the more regenerative explants for induction of shoot and root. Studies of Asparagus officinalis L. clonal propagation could also be efficiency adapted for other crops in future research.

ACKNOWLEDGEMENT

The authors would like to thank Sultan Idris Education University (UPSI) and Research Management and Innovation Centre (RMIC) of UPSI for providing University Research Grant (GPU), project code : 2016-0038-102-01 and the facilities to carry out this research.

REFERENCES

[1]. Prophens, J., Nuez, F. & Carena, M. J. (2008). *Handbook of Plant Breeding*. Springer Publishing, pp: 364.

[2]. Kanno, A. & Yakoyama, J. (2011). *Asparagus. In Wild Crop Relatives: Genomic and Breeding Resources, Vegetables. Kole C. (ed)*. Springer - Verlag, Berlin, Heidelberg, 3: 23 - 42.

[3]. Stajner, N., Bohanee, B. & Marijana, J. (2002). *In Vitro* Propagation of *Asparagus maritimus* - a rare Mediterranean Salt - Resistant Species. *Plant Cell Tiss. Org*., 70: 269 - 274.

[4]. Falloon, P. G. & Nikoloff, A. S. (1986). Asparagus: Value of Individual Plant Yield and Fern Characteristic as Selection Criteria. *New Zealand J. Exp. Agric*. 14 (4), 417 - 420.

[5]. Gonzalez Castanon, M. L. (1990). Evaluation of Male and Female Asparagus Plants. Interest in Obtaining Male or Dioecious Hybrids. *Acta Hort*. 271, 83 - 89.

[6]. Thomas, D.T and Philip B. (2005). Thidiazuron-induced high frequency shoot organogenesis from leaf-derived callus of a medicinal climber,*Tylophora indica* (Burm.F) *Merrill.In Vitro Cell .Dev.Biol- Plant*. 41:124-128.

[7]. Thomas, T.D. and Jacob, A., (2004). Direct somatic embryogenesis of *Curculigo orchioides* Gaertn. an Endangered medicinal herb. *J. Plant Biotech*., 6 :193-197.

[8]. Rout, G.R., Samantaray, S. and Das, P. (2000).*In vitro* manipulation and propagation of medicinal plants. *Biotechnology Advances*.18:91-120.

[9]. Arora, R. and Bojwani, S.S. (1989). *In vitro* propagation and low temperature storage of *Saussurea lappa* C B Clarke, an endangered medicinal plant. *Plant Cell Rep*.8: 44-47.

[10]. Vishwanath, M.P., and Jayanthi, M. (1997) Micropropagation of two species of *Rauvolfia*(*Apocynaceae*). *Curr. Sci.* 72: 961-965.

[11]. Anis, M. and Faisal, M.(2005). *In vitro* Regeneration and Mass Multiplication of *Psoralea corylifolia*: An Endangered Medicinal Plant. *Ind. J. Biotech*. 4: 261-264.

[12]. Uppendra, D., Sumit, M., and Meena, J. (2005). Organogenesis, embryogenesis, and synthetic seed production in *Arnebia euchroma* - a critically endangered medicinal plant of the Himalaya. *In Vitro Cell. Dev. Biol. Plant.* 41: 244-248.

[13]. Murashige, T. & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures. *Physiologia Plantarum* 15: 473 – 497.

[14]. Patel, L. S. & Patel, R. S. (2015). Rapid *In Vitro* Micro Propagation of *Asparagus racemosus* Willd. from Nodal Explants. *Int. J. of Current Microbiology and Applied Science*, Vol. 4. pp. 607-617.

[15]. Sarabi, B. & Almasi, K. (2010). Indirect Organogenesis is Useful for Propagation of Iranian Edible Wild Asparagus (*Asparagus officinalis* L.). *Asian Journal of Agriculture Sciences* 2(2): 47-50.

[16]. Abbasi, B. H., Rashid, A., Khan, M. A., Ali, M., Shinwari, Z. A., Ahmad, N. & Mahmood, T. (2011). *In Vitro* Plant Regeneration in *Sinapis alba* and Evaluation of its Radical Scavenging Activity. *Pak. J. Bot*., 43: 21-27.

[17]. Debergh, P.C. and Maene, I.J. (1981). A scheme for commercial propagation of ornamental plants by tissue culture. *Sci. Hort*. 14: 335-345.

[18]. Read, P. E. (1990). *Environmental effects in micropropagation in Y. P. S. Bajaj(eds.), Handbook of plant cell culture. Vol 5. Ornamental species.* New York: Macmillan Publishing Company.

[19]. Sagare, A. P., Lee, Y. L., Lin, T. C., Chen, C. C. & Tsay, H. S. (2000). Cytokinin-Induced Somatic Embryogenesis and Plant Regeneration in *Coryodalis yanhusuo* (*Fumariaceae*) – a Medicinal Plant. *Plant Sci*. 160: 139-147.

[20]. Rout, G. R., Mohapatra, A., & Mohan J, S. (2006). Tissue culture of ornamental pot plant: a critical review on present scenario and future prospects. *Journal Biotechnology Advanced* (JBA-06036). doi:10.1016/j.biotechadv.2006.05.001

[21]. Rajkarnikar, K. M., Sainju, H. K. & Bhatta, G. D. (2000). *In Vitro* Culture of *Rouwolfia serpentine* L. Benth. Ex. Kurz. Proc. Nepal *Japan Joint Symp*. 232-234.

[22]. Ranjitkar, K. M., Bhatt, G. D. & Adhikari, M. K. (2004). *Micropropagation of Swertia cliata. In: Scientific Publication Bull Dept. Plant Res. No. 24. Natural Product Research Laboratory*, Nepal: Thapathali, Kathmandu. 10-12.

[23]. Paudel, N., Paudel, L. P. & Rai, P. D. (2017). Callus Induction and Shoot Regeneration of *Lycopersicon esculentum* L. *Int. J. Sci. Res.* 6(8): 1346-1348.

[24]. Pant, Krishna, K. & Joshi, S. D. (2009). Direct Multiple Shoot Induction from the Seed Explants of Wild Nepalese *Asparagus racemosus* Wild. *Plant Arch*. 9(1): 443-446.

[25]. Aryal, M. R., Paudel, N. & Ranjit, M. (2018). Elimination of Chhirkey and Foorkey Viruses from Meristem Culture of Large Cardamom (*Amomum subulatum* Roxb.). *Eur Online J. Nat. Social Sci*. 7(2): 424-442.

[26]. Hasancebi, S., Kara, N. T., Cakir, O. & Ari, S. (2011). Micropropagation and Root Culture of Turkish Endemic *Astragalus chrysochlorus* (laguminosae). *Turk. J. Bot.* 35: 203-210.

[27]. Theim, B. (2003). *In Vitro* Propagation of Isoflavone- Producing *Pueraria lobata* (Willd.) Ohwi. *Plant Science* 16595): 1123-1128.

[28]. Azad, M. A. K., Amin, M. N. & Begum, F. (2003). Rapid Clonal Propagation of a Medicinal Plant-*Adhatoda vasica* Nees using Tissue Culture Technique. *Online J. Biol. Sci*. 3(2): 172-182.

[29]. Shigeta, J., Sato, K., Tanaka, S., Nakayama, M. & Mii, M. (1996). Efficient Plant Regeneration of Asparagus by Inducing Normal Roots from *In Vitro* Multiplied Shoot Explants Using Gellan Gum and Glucose. *Plant Sci*. 113(1): 99-104.

[30]. Sauer, M., Jakob, A., Nordheim, A. & Hochholdinger, F. (2006). Proteomic Analysis of Shoot-borne Root Initiation in Maize (*Zea Mays* L.). *PROTEOMICS* 6(8): 2530-2541.