ISSN No.: 2454- 2024 (online)

International Journal of Technical Research & Science

IN VITRO MORPHOGENESIS OF SHOOT INDUCED CALLUS OF ASPARAGUS RACEMOSUS WILLD. INTO SOMATIC EMBRYOIDS

Krishna Kumar Pant[1], Sanu Devi Joshi[2]
E-Mail Id: krishna.k.pant@gmail.com
Central Dept. of Botany, Tribhuvan University, Kirtipur, Kathmandu, Nepal

Abstract- Asparagus racemosus Willd. locally known as "Kurilo" or "Shatavari" belonging to the family Liliaceae is a multipurpose plant facing severe threat have been studied for the purpose of its conservation using tissue culture as a promising technique. Here we studied different types of calli formed from the nodes on MS medium containing different concentrations of auxins and cytokinins and observed the induction of somatic embryoids at different stages of development from those calli. The calli with somatic embryoids were subcultured to regenerate normal seedlings which were later rooted in vitro and finally acclimatized.
Keywords: Shatavari, Asparagus racemosus, in vitro, somatic embryoids, acclimatization.

1. INTRODUCTION

Asparagus racemosus Willd. locally known as "Kurilo" or "Shatavari" belongs to the family Liliaceae. It is an undershrub with highly branched woody stems, growing up to 2m in height. The fleshy tuberous roots are 30-100 cm long and 1-2 cm thick in bunch attached at the stem base. The leaves are reduced to small scales or needle-like spines called cladodes. The flowers are small, white, fragrant and in simple or branched racemes. When the plants are young, stems are very delicate, brittle and smooth. Its fruits are globular or obscurely 3-lobed, pulpy berries, that are purplish black when ripe; its seeds have hard and brittle testa. This plant can be found growing naturally in the tropical and sub-tropical forests throughout Nepal up to 1500 m above sea level and is also distributed in India, Malaysia, Australia and Africa [1]. Altogether eight species of this plant have been reported from Nepal including a racemosus.

It is widely used for multiple purposes, and its medicinal importance has been recognized by Ayurveda for centuries. Although almost all parts of this plant have some medicinal properties, roots and young shoots are of higher significance. Young spears are consumed as vegetable or salad and are considered as a balanced health food with many essential nutrients. Traditionally and in Ayurveda the tuberous roots are used mainly to promote milk secretion and disorders of female genitourinary tract; as a styptic and ulcer healing agent, intestinal disinfectant, astringent in diarrhea, nervine tonic, sexual debility for spermatogenesis, gout, puerperal diseases, haematuria, bleeding disorders, hyperacidity, demulcent, diuretic, aphrodisiac, tonic, alterative, antiseptic, antidiarrheal, glalctogogue and antispasmodic [2]. It is also used to treat debility, especially in women, and infertility, impotence, menopause, stomach ulcers, hyperacidity, dehydration, lung abscess, haematemesis, cough, herpes, leucorrhoea and chronic fevers, delay ageing process and form health food ingredients in several Ayurvedic formulations [3].

Recently, many active compounds like steroidal saponins, shatavarins I–IV (phytoestrogens) [4,5,6 and 7], aglycones, alkaloids like asparagin- an anticancer agent [8] and many other active pharmacologically important compounds have already been isolated from the roots of this species. Leaves contain rutin, diosgenin and a flavonoid glycoside identified as quercetin - 3 - glucuronide. Flowers contain quercetin hyperoside and rutin. Fruits contain glycosides of quercetin, rutin and hyperoside and steroidal saponins [9]. This species especially its roots have been used as medicine since the Vedic time centuries ago (Ayurveda). Recent scientific investigations have confirmed and increased importance of this species in various medicines. Hence this species can be considered wholly a medicine having potential to cure different diseases. These studies have further strengthened the traditional medical knowledge with scientific bases.

This species is in high trade from the forests of Nepal to the international markets. Asparagus racemosus from Nepal is highly traded in India because the ayurvedic physicians prefer the roots of A. racemosus from Nepal (pale brown slightly resinous) as it is more effective than the Indian ones and have also been confirmed that the main source of A. racemosus in India is Nepal [6 and 10]. To meet the demand, a large volume of this species is collected annually from different parts of the country and sold in both local as well as international markets. The exact data however are not available. To overcome this threat of conservation, a reliable and rapid method of mass multiplication of this species is necessary.

Very few works on this species have been previously done and mainly with other species of the genus and focus mainly on shoot multiplication through nodal bud [11]. Some examples of works that have been done in other species are: A. densiflorus on factors influencing regeneration from protoplast [12]; plant regeneration in A. verticillatus [13]; direct multiple shoot induction in A. adscendens [14]; and some publications on somatic embryogenesis from the callus cultures of A. officinalis [15-20]. In the present study, we are trying to work on the wild Nepalese species.

2. MATERIALS AND METHODS

The seeds of Asparagus racemosus were collected from a local garden of Sim Gaun, Kirtipur, Kathmandu (approx. 1400 m asl). To produce sterile explants for the experiment, the healthy seeds were selected and treated with liquid detergent for 15 minutes and was washed under running tap water for 45 minutes. After this the seeds were treated with 90% ethyl alcohol for 5 minutes and washed with distilled water. Finally, the seeds were treated with 0.2% mercuric chloride for another 5 minutes and washed with sterile water four times under the laminar air flow hood before inoculation in the hormone free (MS) medium [21]. The nodes of in vitro germinated seedlings from the seeds on Hormone free MS media were used as explants. Nodes were excised and pieces of about 0.5-1cm were inoculated on the MS medium containing MS basal salts, 3% sucrose, 100mg myo-inositol, 0.8% agar and different concentrations of a-naphthaleneacetic acid (NAA) and 6- benzylaminopurine (BAP) and NAA and Kinetin (Kin) either singly or in combinations for various responses in glass tubes (150mm × 25mm containing approx. 12 ml media) and jam bottles (approx. 16.5 ml media) inside the laminar air flow cabinet. The concentration ranges for all the hormones used singly in the media were 0.1, 0.5, 1.0 and 2.0 mg-l., similarly, in combination of NAA and BAP and NAA and Kin, NAA concentration was limited up to 1.0 mg-l. whereas BAP and Kin. up to 2.0 mg-l. The media were adjusted to pH 5.8 with 0.1 N KOH/HCl and autoclaved at 121°C and 15lb pressure for 20 minutes. They were cultured under illuminated condition of 16-hour photoperiod using cool white fluorescent lamps at 25oC ±1oC. The results were observed and recorded in every week. The calli formed from the first culture were sub cultured in the same media for further response after 6-12 weeks depending upon the response period. In each case a total of 6 replications were used for each treatment and the experimental trials were repeated three times.

A fraction of the calli of different durations (6-12 weeks) was studied under compound microscope. For microscopic studies the calli were dipped in 1% acetocarmine for 12 hours and heated with it in the test tube until boil. The callii then were squashed and analyzed under the various magnifications of a compound microscope for the study of different stages of somatic embryoids.

The remaning embryoids along with calli were sub cultured to germinate in vitro and the multiple shoots obtained were rooted in vitro using different auxins. Thus obtained seedlings were finally transferred to 100% coco peat, 100% sand and 50% each coco peat and sand mixture in vivo. They were kept in the plastic shade and were regularly observed and watered at an interval of 3-5 days depending upon the moisture on the bed. All the physical conditions were kept constant throughout the experiment. The callus induction period, callus mass, presence of somatic embryoids, SE germination, rooting and acclimatization status was recorded, calculated and data were analyzed.

3. RESULT AND DISCUSSION

3.1 Callus Induction

Most of the treatments except control and few others induced callus but not all the calli induced were embryogenic. From the present study we conclude that the callii of 10-12 weeks are good enough for the study of somatic embryogenesis (data not shown). For the induction of somatic embyroids, the quality of the callus is important. As observed in the experiment friable, tough and greenish yellow calli were good source of embryoids whereas the watery, brown and black callii were non embryogenic [22].

3.2 Somatic Embryogenesis

In MS + NAA 2.0 mg/l, most of the concentrations of BAP or Kn and many other auxin NAA + Cytokinin (BAP or Kn) combinations induced somatic embryoids from the callus. As treated individual, BAP was found to induce somatic embryoids more than Kinetin in the medium. In the same way we observed maximum number of mature somatic embryoids from BAP 2.0 mg/l, NAA 0.1 + BAP 1.0 and NAA 0.1 + BAP 2.0 mg/l (Fig. 1 and 2). The plantlets germinated from the somatic embryoids in general were found to be relatively much thicker and with less cladodes than the normal shoots (Fig. 3 and 4). Similarly, the length of internodes was also longer than the normal ones. This might be because of the presence of somatic variability and needs further investigation (data not shown). A very few or no literatures are available on the somatic embryogenesis of this species probably because this species is less known to the western world. The previous works on somatic embryogenesis induction in A. officinalis have

been studied widely by various workers [23; 17; 24; 25; 26 and 27] using shoot explant induced calli of different varieties of A. officinalis either without any addition of any hormone or incorporating small amout of NAA (0.1 mg/l) + Kn or other cytokinin in the MS medium. In our case, (NAA 0.1 + BAP 2.0 mg/l) the somatic embryoids grew so much that they were easily visible (Fig. 3.2).

Fig. 3.1 Fig. 3.2 Fig. 3.3 Fig. 3.4

Fig. 3.1-3.4: Somatic Embryoid Induction and Germination

Fig. 3.1- Induction of somatic embryoids from the friable callus induced from the node on MS + NAA 0.1 + BAP 1.0 mg/l after 12 weeks of culture.

Fig- 3.2- Formation of somatic embryoids from the node induced callus on MS + NAA 0.1 + BAP 2.0 mg/l after 13 weeks of culture.

Fig. 3.3- Germination of embryoids after 8 weeks on MS + IBA 1.0 + BAP 1.0 mg/l

Fig. 3.4- Germinated embryoids after 8 weeks on NAA 0.1 mg/l

The embryogenic calli when spread appeared densely nucleated cells (Fig. 5). Similarly, actively dividing embryogenic cells at various stages of somatic embryoids like 2 celled (Fig. 6), 4 celled and multicellular proembryoids (8 or more cells) of different shapes were observed. The embryogenic cells generally were found to divide transversely to give a linear four celled pro embryoids (Fig. 7 and 8). Like a typical monocot pro embryo, a long suspensor was also observed (Fig. 9). It has also been observed that the dense apical cell (Fig. 10) then divides vertically and transversely to form an octant (Fig. 11 and 12). Later this converts into an early globular (Fig. 13), mid globular (Fig. 14) pro embryo structure. After a repeated division it attains a transition stage (torpedo) of embryonic development (Fig. 15 and 16). Finally, a heart stage of the somatic embryo was observed with a distinct cotyledon (Fig. 17).

Finally, most of the media including MS basal were able to germinate the somatic embryoids. The media containing 0.1-1.0 mg/l auxin helped to induce roots in the plantlets. The survival percentage of these plantlets in the outer environment was however very low (>10%) as compared to the multiple shoots induced from nodal explants (60%).

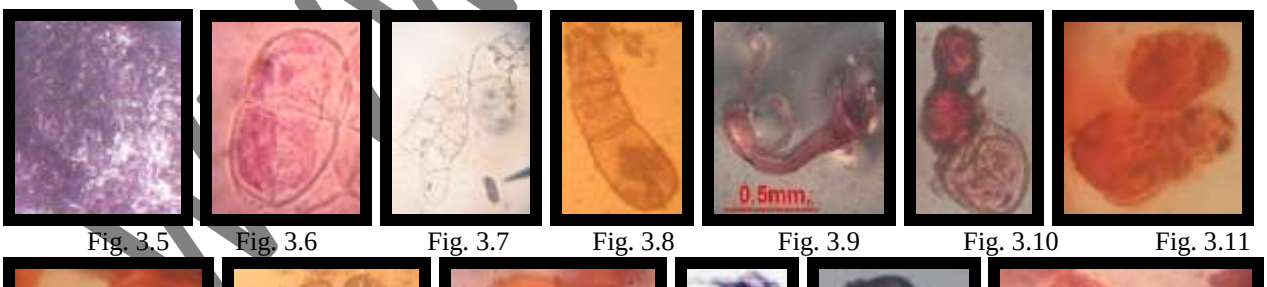

Fig. 3.5 Fig. 3.6 Fig. 3.7 Fig. 3.8 Fig. 3.9 Fig. 3.10 Fig. 3.11

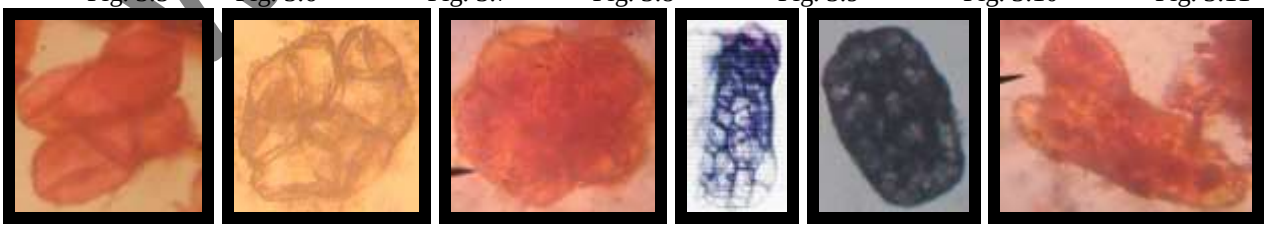

Fig. 3.12 Fig. 3.13 Fig. 3.14 Fig. 3.15 Fig. 3.16 Fig. 3.17

Fig. 3.5-3.17 Photomicrographs of Different Stages of Somatic Embryoid

Fig. 3.5- Embryogenic cells of callus spread (12 weeks) from MS + BAP 1.0 mg/l (10×4).

Fig. 3.6- Actively dividing embryogenic cells of the callus (10×40).

Fig. 3.7 and 3.8- Longitudinal cell divisions forming 4 celled pro embryoids (10×40).

Fig. 3.9- A pro embryoid with a long suspensor.
Fig. 3.10- Densely nucleated apical cell of the pro embryoid.
Fig. 3.11-3.12- Transverse and vertical divisions to give octant pro-embryoids (10×40).
Fig. 3.13- Early globular stage of pro embryoid (10×40).
Fig. 3.14- Mid globular stage of pro embryoid (10×40).
Fig. 3.15 and 3.16- Transition (torpedo) stage of somatic embryoids (10×40).
Fig. 3.17- A cotyledonary stage somatic embryoid (10×40).

CONCLUSION

Asparagus racemosus, a potential medicinal as well as vegetable plant can easily be induced to produce a vast number of somatic embryoids which can be used as aritificial seeds. From our initial study it seems that there is a huge possibility of further research in this plant.

ACKNOWLEDGEMENT

We are thankful to Shambhu Ram Bista for providing the seeds and to the Central Department of Botany for providing laboratory facilities. We are also thankful to the University Grants Commission (Nepal) for providing partial funding.

REFERENCES

[1] Joshi, K.K and Joshi, S.D. 2001. Genetic Heritage of Medicinal and Aromatic Plants of Nepal. Buddha Academic Pub., Pp 239.
[2] Goyal, R.K., Singh, J. and Lal, H. 2003. Asparagus racemosus-An Update. Indian Journal of Medical Sciences, 57(9): 407-414.
[3] Kumar, N.M., Abdul Khader, J.B.M., Rangaswami, P. and Irulappan, I. 1997. Introduction to Spices, Plantation Crops, Medicinal and Aromatic Plants. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi.
[4] Hayes, P.Y., Jahidin, A.H., Lehmann, R., Penman, K., Kitching W., and De Voss, J.J. 2006a. Asparinins, asparosides, curillins, curillosides and shatavarins: structural clarification with the isolation of shatavarin V, a new steroidal saponin from the root of Asapragus racemosus. Tetrahedron Letters, 47: 8683-8687.
[5] Hayes, P.Y., Jahidin, A.H., Lehmann, R., Penman, K., Kitching W., and De Voss, J.J. 2006b. Structural revision of shatavarins I and IV, the major components from the roots of Asparagus racemosus. Tetrahedron Letters, 47: 6965-6969.
[6] Khare, C.P. 2007. India Medicinal Plants an Illustrated dictionary. Springer-Verlag Berlin/Heidelberg. Pp: 750.
[7] Hayes, P.Y., Jahidin, A.H., Lehmann, R., Penman, K., Kitching W., and De Voss, J.J. 2007. Steroidal saponins from the roots of Asparagus racemosus, Phytochem.doi: 10. 1016/ Journal of phytochemistry, 2007.09.001.
[8] Joy, P.P., Thomas, J., Mathew S., and Skaria, B.P. 1998. Medicinal Plants. Kerala Agriculture University, Aromatic and Medicinal Plants Research Station, India, pp: 38.
[9] Mandal, D., Banerjee, S., Mondal, N.B., Chakravarty, A.K. and Sahu, N.P. 2006. Steroidal saponins from the fruits of Asparagus racemosus. Phytochemistry, 67: 1316-1321.
[10] Puri, H.S. 2003. Rasayana: ayurvedic herbs for longevity and rejuvenation. Taylor and Francis, London, ISBN 0-415-28489-9. (Page: 255)
[11] Kar, D.K. and Sen, S. 1985. Propagation of Asparagus racemosus through tissue culture. Plant Cell, Tissue and Organ Culture, 5(1): 89-95.
[12] Benmoussa, M., Mukhopadhyay, S. and Desjardins, Y. 1997. Factors influencing regeneration from protoplasts of Asparagus densiflorus cv. Sprengeri. Plant Cell Reports, 17(2): 123-128.
[13] Ghosh, B. and Sen, S. 1998. Plant Regeneration in Asparagus verticillatus L. J. Herbs, Spices and Medicinal Plants, 4(1): 9-17.
[14] Mehta, S.R. and Subramanian, R.B. 2005. Direct In vitro Propagation of Asparagus adscendens Roxb. Plant Tissue Culture, 15(1): 25-32.
[15] Levi, A. and Sink, K.C. 1991. Somatic embryogenesis in asparagus: the role of explants and growth regulators. Plant Cell Reports, 10: 71-75.
[16] Saito, T., Nishizawa, S. and Nishimura, S. 1991. Improved culture conditions for somatic embryogenesis from Asparagus officinalis L. using an aseptic ventilative filter. Plant Cell Reports, 10: 230-234.
[17] Levi, A. and Sink, K.C. 1992. Asparagus somatic embryos: Production in suspension culture and conversion to plantlets on solidified medium as influenced by carbohydrate regime. Plant Cell, Tissue and Organ Culture, 31: 115-122.
[18] Li, B. and Wolyn, D.J. 1997. Interactions of ancymidol with sucrose and a-naphthaleneacetic acid in promoting asparagus (Asparagus officinalis L.) somatic embryogenesis. Plant Cell Reports, 16: 879-883.

[19] Kunitake, H., Nakashima, T., Mori, K. and Tanaka, M. 1998. Somaclonal and chromosomal effects of genotype, ploidy and culture duration in Asparagus officinalis L. Euphytica, 102: 309-316.

[20] Limanton-Grevet, A. and Jullien, M. 2000. Somatic embryogenesis in Asparagus officinalis can be an in vitro selection process leading to habituated and 2,4- D dependent embryogenic lines. Plant Physiol. Biochem., 38(7-8): 567-576.

[21] Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, **15**: 473-497.

[22] Pant, K.K. and Joshi, S.D. 2009. In vitro Multiplication of Wild Nepalese Asparagus racemosus Through Shoots and Shoot Induced Callus Cultures. Botany Research International 2 (2): 88-93.

[23] Kohmura, H., Chokyu, S., and Ikeda, Y. 1990. Micropropagation of Asparagus through Somatic Embryogenesis. Bulletin of Hiroshima Pref. Agricultural Experiment Station, 53: 43-50.

[24] Odake, Y., Udagawa, A., Saga, H. and Mii, M. 1993. Somatic Embryogenesis of Tetraploid Plants from Internodal Segments of a Diploid Cultivar of Asparagus officinalis L. Grown in Liquid Culture. Plant Science, 94: 173-177.

[25] Delbreil, B., Goebel-Tourand, I., Lefrancois, C.and Jullien, M. 1994. Isolation and characterization of long-term embryogenic lines in Asparagus officinalis L. Journal of Plant Physiology, 144: 194- 200.

[26] Li, B. and Wolyn, D.J. 1995. The Effect of ancymidol, abscisic acid, uniconazole and paclobutrazol on somatic embryogenesis of Asparagus. Plant Cell Reports, 14: 529-533.

[27] Li, B. and Wolyn, D.J. 1996. Temperature and Genotype affect Asparagus Somatic Embryogenesis. In Vitro Cell Biology- Plant, 32:136-139.