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In vitro Multiplication of Wild Nepalese *Asparagus racemosus* Through Shoots and Shoot Induced Callus Cultures

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Abstract: For the conservation of high value but neglected medicinal plant (Asparagus racemosus) of Nepal an effort has been made to multiply its number rapidly using tissue culture technique. The plant has been successfully multiplied. For the multiplication, all possible explants including callus have been used and the callus and shoot explants (shoot tips and nodes) played significant role. Mainly four parameters: callus, buds, shoots and roots inductions were studied. NAA singly played a good role in all parameters except bud induction. Similarly, BAP played good roles in shoot and bud inductions, whereas combinations of NAA and BAP at various levels were found to be effective in almost all cases. The multiplied adventitious shoots were successfully rooted (*in vitro* and *in vivo*) using NAA, acclimatized and transferred to natural conditions.

Abbreviations: BAP: 6-benzylaminopurine; MS: Murashige and Skoog (1962); NAA: *a* naphthaleneacetic acid; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; 2,4-D: 2, 4 Dichlorophenoxyacetic acid and Kin: Kinetin.

Key words: Acclimatization · Bud induction · Callus culture · Explants · Medicinal plant · Nepal

INTRODUCTION

Asparagus racemosus Willd. locally known as "Kurilo" or "Shatavari" belongs to the family Liliaceae. It is an undershrub climber with extensively branched woody stems, growing up to 2m in height. The succulent 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.

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 the roots are used mainly to promote milk secretion and as a demulcent, diuretic, aphrodisiac, tonic, alterative, antiseptic, antidiarrheal, glalctogogue and antispasmodic [1]. 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 [2].

Using the modern scientific tools many active compounds like several steroidal saponins [3-5], aglycones, alkaloids like *asparagin*-an anticancer agent [6] 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 [7] while fully ripe fruits contain cyanidin-3-galactoside and cyanidin-3-glucorhamnoside. These studies have further strengthened the traditional medical knowledge with scientific bases.

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Sixty percent of the world population and 80% of the population in developing countries rely on traditional medicine, mostly plant drugs, for their primary health care needs [8]. An account of 70% of the population of India [9], 80% of Pakistan [10] and 80% of Nepal are dependent on traditional plant based medicines [11]. These figures are increasing every year because of their safe mode of action in human health. Due to above mentioned importance this species is in threatened condition and is listed by as the plant of high economic importance by the government of Nepal. Local healers and local as well as cross border traders are rampantly collecting this species from the wild which is causing a severe threat to its existence in Nepal. Most of this species' industrial requirements for production of different commodities are met through wild collections from forests. Very little effort has been made to conserve this species. Conservation of this species has become an immediate need of the country. If behaviors are not modified, after a couple more years, there will be no species left to conserve. For the conservation of this species, rapid multiplication and rehabilitation in its natural habitat is necessary. To overcome this threat, a reliable method of quick multiplication like tissue culture (conventional vegetative propagation is a very slow and laborious) and methods of *in situ* as well as *ex situ* conservation could well provide a viable solution to the problem [12].

Very few works on this species have been previously done and mainly with species of other countries with completely different climatic conditions and a focus on shoot multiplication through nodal bud [13]. Some examples of works that have been done in other species are: *A. densiflorus* by [14] on factors influencing regeneration from protoplast; plant regeneration in *A. verticillatus* by [15]; direct multiple shoot induction in *A. adscendens* by [16]; and lots of publications on somatic embryogenesis from the callus cultures of *A. officinalis* [17-22]. In the present study, we are trying to work on the wild Nepalese species.

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 that sank in the water 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 [23] (MS) medium.

The different parts of *in vitro* germinated seeds on Hormone free MS media and grown seedlings of *Asparagus racemosus* were used as explants for all kinds of experiments to make sure that the explants have no residual effects of hormone from the previous culture conditions. Different parts like shoot explants (nodes, internodes, shoot tips), cladodes, roots callus 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; similarly, different concentrations of auxins, IAA, IBA and 2,4-D for various responses in glass tubes (150mm × 25mm) and jam bottles with approximately 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 callus or multiple shoots formed from the first culture were sub cultured either in the same media for further response or shoot and root inducing media separately after 6-8 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. Multiple shoots obtained from the sub-culture of callus and shoot explants in different hormone media were detached and were either cultured *in vitro* in the rooting medium containing different concentrations of IAA, IBA and NAA or pulse treated with 100mg/l of the same hormones and 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. *In vivo* rooting response and hardening responses were recorded in the plastic house. All the physical conditions were kept constant throughout the experiment. The callus induction period, callus mass, number of shoots and roots/explants (shoot tip, nodes and callus mass) and acclimatization status were recorded calculated, data were analyzed using

analysis of variance (SPSS 11.5 for windows) and the mean comparison was done by a least significant difference test at the 5% and 1% level of probability.

RESULTS

The shoot tips, nodes and internodes have shown single or multiple shoot formation on MS hormone free medium (Fig. 15) whereas roots, cladodes and callus showed no effect of any kind in any of the media used throughout the experiment. These shoots on sub culture grew well and branched. MS hormone free media showed no response of any kind in callus induction, bud formation and root initiation with all the explants. Most of the cultures in hormone media induced callus in the beginning.

NAA singly showed an increase in callus induction from nodes, internodes and shoot tips. The callus induction trend was found to be gradually decreasing with an increase in concentration except at 2.0 ppm. In callus culture NAA 0.1 ppm was found to be excellent in multiple shoot induction with an average of 6 shoots/explants and the shoots were very healthy (Fig. 1&2), whereas other higher concentrations showed callus growth and root induction (Fig. 3 and 4). Other concentrations above 0.1 ppm showed multiple shoot induction except 0.5 ppm. from the shoot explants, but in a decreasing rate. Low concentration of NAA i.e. 0.1 ppm showed some bud initiation at the nodes which was also observed in 2.0 ppm but in a lower rate. These buds failed to develop in the form of shoots and finally gave callus at later stages. The number of roots induced /explants was recorded to increase with an increase in concentration but the root thickness gradually decreased with an increase in concentration of hormone. At the same time at higher concentration of NAA callus at the base of shoot was found to be persistent causing the late and weak growth of shoots. Other auxins like IAA and IBA showed almost no response of any kind with this species whereas 2,4-D showed some callus induction which was not very remarkable.

BAP singly showed very little or no effect in callus induction and root formation from all the explants. The higher concentrations showed and increase in bud formation with an average of 1 bud per node at 2.0 ppm which did not grow further whereas the buds induced in low concentration i.e 0.1 and 0.5 ppm gave callus and multiple shoots with an average of 6.85 and 8 shoots/ explants respectively. This number gradually decreased as the concentration increased, they instead gave multiple abnormal flat, thick leafy and branched shoot like structures. The callus cultures also responded similarly as the other shoot explants with different concentrations of BAP (Fig. 5, 6, 7 and 8).

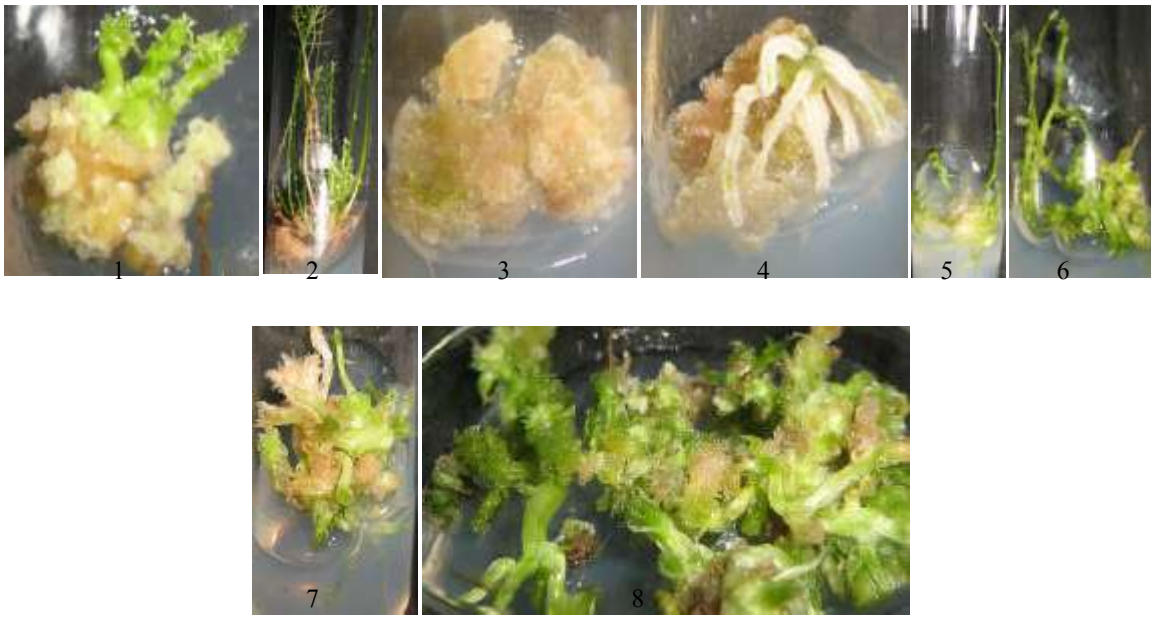
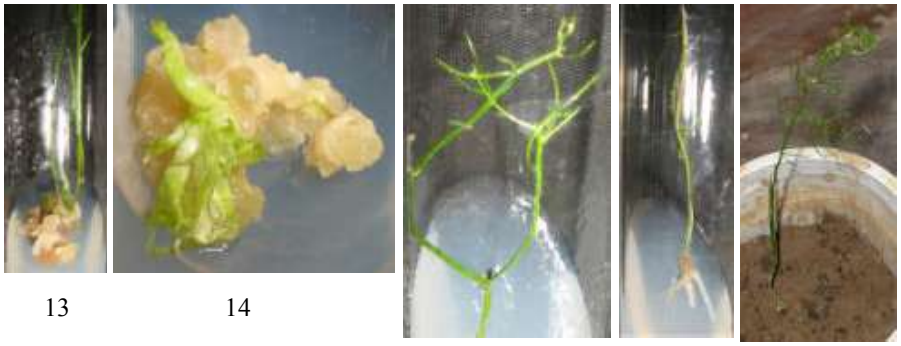


Fig. 1-4:Callus induction and multiple shoot/ root formation with NAA 0.1, NAA 0.1, NAA 1.0 and NAA 2.0 respectively
Fig. 5-8: Multiple normal as well as abnormal shoots with BAP 0.1, BAP 0.1, BAP 0.5 and BAP 2.0 respectively

9 10 11 12



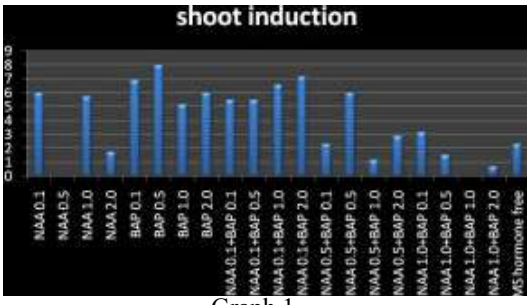
13 14 15 16 17

Fig. 9-14: Multiple bud, root and shoot induction both from the node as well as callus cultures with NAA and BAP in combinations (NAA 0.1+BAP 0.5, NAA 0.1+BAP 1.0, NAA 0.5+BAP 0.5, NAA 0.5+BAP 1.0, NAA 1.0+BAP 0.1 and NAA 1.0+BAP 2.0 respectively).

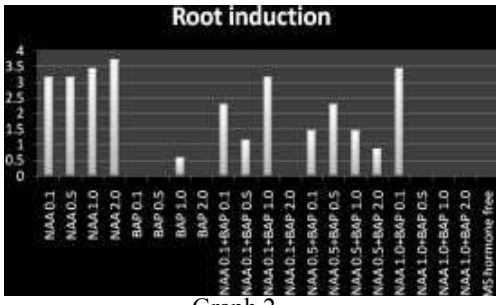
Fig. 15: Multiple shoots from a node in basal MS medium.

Fig. 16: Rooting of micro shoot with NAA 0.1.

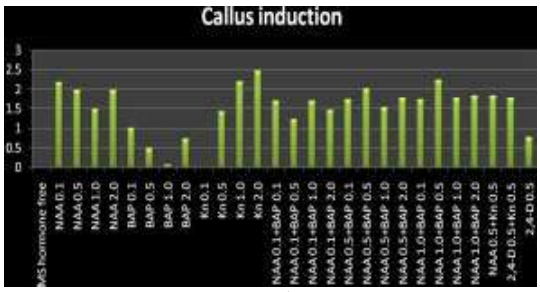
Fig. 17: Acclematized shoot in garden soil after 3 months.



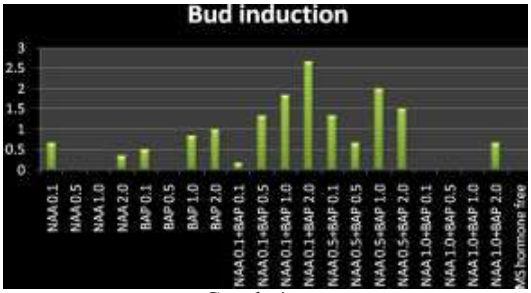
Graph 1



Graph 2



Graph 3



Graph 4

Scale for Graph 1: (0-2 = Low, 2-4 = medium, 4-6 = high and 6-8 = very high)

Scale for Graph 2-4: (0-1 = Low, 1-2 = medium, 2-3 = high and 3-4 = very high)

In combination of BAP and NAA, callus induction was found to increase with an increase of the concentration of NAA from 0.1 ppm to 1.0 ppm but the role of BAP was observed to be of very little or no significance. In general, almost all the concentrations of BAP and NAA in combination initiated callus. In bud formation, the trend was just opposite to callus induction i.e. an increase in BAP concentration showed increase in the number of buds per explant until the NAA concentration was limited up to 0.1 and 0.5 ppm (Fig. 10 and 12) but as the concentration of NAA was increased to 1.0 ppm and above the bud formation drastically dropped to almost zero. The callus cultures at these concentrations of NAA gave multiple shoots, buds, abnormal buds and leafy shoots with 0.1, 0.5, 1.0 and 2.0 ppm of BAP respectively. The multiple shoots were induced mainly at lower concentration combinations of both the hormones (Fig. 9 and 11). When the concentration of NAA was increased to 1.0 ppm., at lower concentrations of BAP both shoot explants and callus cultures gave few shoots and roots (Fig. 13) but as the BAP concentration was increased gradually, callus induction and a few multiple shoots were observed Fig. 14). Here, root induction was completely blocked although NAA concentration was favorable. Detailed responses of different hormones at different levels are given in Graphs 1-4.

Root induction was favored by all the concentrations of NAA either singly or in combinations. In our experiment, other auxins like IAA and IBA did not show any kind of rooting or other remarkable response both *in vitro* and *in vivo* rooting of the micro shoots whereas NAA was effective only in *in vitro* condition (Fig. 16). The *in vitro* rooted micro shoots survived, grew and branched in the plastic shade house for about 3 months and they gradually died in the winter. The final survival percentage of both *in vitro* and *in vivo* rooted shoots in nature was 60 and 20 respectively (Fig 17).

DISCUSSION

From our experiment data of seed germination, number of shoots per seed and micro shoot survival percentage only it is clear that *in vitro* process is a promising tool for the rapid multiplication of this endangered medicinal species. From the above result it is obvious that NAA at many of the concentrations either singly or in combinations is effective in the induction of callus, shoots and roots which might be because of its chemically stable, low mobility in the plant and prolonged action nature [24]. [13,14 and 16] induced roots on media containing IBA in *A. densiflorous, A. adscendens* and *A racemosus* shoots respectively whereas [15] reported good rooting on MS media containing IAA in *A. verticillatus*. In contrary these two auxins did not show any roots both *in vitro* and *in vivo* in our experiments. [25] used low concentrations of NAA and Kin. Along with gellan gum as the solidifying agent instead of agar to induce storage roots from the shoot explants of *A. officinalis.* BAP singly has very little or no response in the induction of callus and roots whereas Kin. also being a cytokinin is a very good callus inducer and root inducer from the callus sub cultures. These two cytokinins being similar in nature have completely different modes of action in the individual plant species. [14] induced callus and shoots from the callus at 1.0 mg/l. BAP with slight modification in the MS medium. Similarly, [15] reported that the callus induction was affected by combinations of 2,4-D and Kin 2,4-D and BAP and NAA and Kin in *A. verticillatus*. They also observed that the shoot induction was supported by BAP and a low level of NAA. [13] observed the nodal buds by the use of IAA and BAP in *A. racemosus* similar to our finding where BAP singly is acted as a good shoot bud inducer at higher concentrations whereas in multiple shoot induction a lower concentration is favorable. In combination with NAA, BAP promotes shoot bud somatic embryos as well as multiple adventitious shoots at its high concentration and a low NAA levels. Similar observations have been done by [26] using IAA and BAP in the ratio 1:1 or 10:1 in *A officinalis*. Reuther also observed buds from the superficial cells of callus using NAA 1 ppm. and BAP 1 pp. in the LS medium in *A. officinalis* similar to our observation using MS media supplemented with 0.5 or 1.0 ppm of NAA along with 2.0 ppm of BAP. [16] reported a very high rate of multiple shoots from nodes of *A. adscendens* using 0.27 µM NAA, 0.46 µM Kin and 0.6% agar.

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