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HIGH FREQUENCY PLANT REGENERATION FROM FULLY MATURE SHOOT PORTION OF *NARDOSTACHYS GRANDIFLORA* DC.

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Abstract

Plants safeguarding through ex situ conservation is a widely accepted approach for effective regeneration, restoration, and conservation of endangered and rare species. Nardostachys grandiflora DC. is a perennial, dwarf, rhizomatous medicinal and aromatic herb belonging to caprifoliaceae family which grows mostly in humid, high elevated slopes between 3000-5000m asl of alpine areas of the North Western Himalayan region. The species is becoming endangered due to unscientific over exploitation indirectly by pharmaceutical and therapeutical drug companies yet there are many other reasons like biotic and abiotic interference. The survival percentage is low in both seedlings and rhizome cuttings due to desiccation problems during the winter and short-snow-free season. The present study aims at the point of conservation, restoration through producing mass scale of plants using the shoot cuttings obtained from the plants before the onset of senescence and to determine the effect of different concentrations of PGR's (IAA, IBA NAA and BAP) on N. grandiflora. The study revealed that lower concentration of IBA (50 µM) showed best result compared to other PGR's, which clearly indicates that for fast regeneration and from the point of conservation view this species shoot cutting is quite beneficial as by using this technique it gives instant growth measures as compared to conventional seed method, without disturbing the mother plants before senescence period and help to reestablish plants in their natural habitat.

Keywords*: Nardostachys grandiflora; Propagation; Regeneration; Cultivation; Conservation*

Introduction

Alpine plants are very sensitive to environmental factors, short growth period, sudden weather changes leads to them specific adaptation approach for cultivation as compared to other lowland species [1]. The *ex-situ* conservation of threatened species are widely accepted strategies by international treaties, conservation and legislation authorities. The Convention on Biodiversity Conservation also recommends s*ex-situ* conservation of threatened plant species [2]. In perennial herbaceous plants underground storage parts like- rhizomes, roots, bulbs etc. withstand long periods of unfavorable environmental conditions and show a slow recovery rate of population, hence required efficacious cultivation practices [3, 4]. *Nardostachys grandiflora* DC is a perennial, dwarf, rhizomatous medicinal and aromatic herb that belongs to *Caprifoliaceae* family, which is naturally found in humid, rock surfaces between 3000-5000m asl of alpine areas of the northwestern Himalayan region. The rhizome contains essential oil which is used for hair-related disorders, insomnia, hypertension, improve neurological function, and making incense sticks, dhoop, perfume etc. [5-7].

Due to unsustainable exploitation of rhizomes which possess essential oil is used for pharmaceutical and therapeutically medicinal and aromatic use, habitat destruction and both

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biotic and a biotic factor involvement are leading this species to critically Endangered. Previously both *ex-situ* and *in-situ* conservation and multiplication efforts of this species have been given by several researchers [5, 8, 9]**.**

Successful conservation, management and restoration of endangered and rare species can be deliberated through variability analysis [10]. It is very important to improve propagation methods for safeguarding over exploited alpine species to encourage cultivation [11]**.** As the growth hormones, auxins and cytokinins stimulate the rooting, enhance cell elongation, growth and biomass and are widely used in the vegetative propagation of plants. As this is a perennial species hence it requires more than three years for maturation if we go through the seed germination method. As this is a perennial species hence it requires more than three years for maturation if we follows the seed germination method. As in the recent years the govt. of India is continuously focusing on farmers/growers economy for doubling the farmer's income (DFI) strategy [12], in case of high altitude farmers this shoot cutting seems to be more beneficial rather than the conventional seed method. As for many alpine species the most conventional and quick method for cultivation is through vegetative method than as compared to sexual method [13].

In this text, result of various PGR's was determined by *ex-situ* conservation through shoot cuttings for effective restoration, conservation and conventional methods of this species.

Material and methods

The aboveground part of *N. grandiflora* plants senescence in the month of October–November in their natural habitats and the plants sprout in next year from belowground rootstock in the month of May-June. We have collected shoot cuttings (top edge) of *N. grandiflora* just before senescence phase starts from alpine field station Tungnath (TN 3400m asl), located between 30°14' N latitude and 79°13' E longitude in Rudraprayag district of Uttarakhand Himalaya, India. After collection top edge cuttings of 7-8cm Length; 4-6mm Diameter and 2-3g weight were prepared from mature plants and further propagated in polyhouse conditions at lower altitude, Srinagar Garhwal (550m asl), Uttarakhand (29°26'-31° 28' N and 77° 49'-80° 6' E), India. The collected shoot (twigs) cuttings were dually washed with both tap and distilled water and then treated in different concentrations (50, 100, 500 and 1000µM) of auxins (IAA, IBA and NAA) and cytokinin (BAP) for 24 hours.

Pre-treated cuttings were planted in Styrofoam trays containing mixture of soil, sand, farmyard manure (FYM), and forest litter (FL) at the ratio of 1:1:1:1. All the Styrofoam trays containing cuttings were kept inside the greenhouse condition at Srinagar (550m asl) for further observations. Each treatment concentration was used in triplicate, and each replicate contained 15 cuttings. The emerged cuttings were transplanted to alpine field nursery at Tungnath after 150 days of planting in polyhouse. The morphological parameters i.e. plant height (PH), number of leaves (NL), leaf area (LA), stem diameter (SD), number of Roots (NR), Length of roots (LR), diameter of roots (DR), and dry biomass (DB)were recorded before plantation to Tungnath.

Raised beds parallel to field were prepared according to the method given by *Nautiyal and Nautiyal (2004)* [14] for this species; the cuttings were transplanted at 30×30cm apart according to their respective treatments [15]. After 300DAP in polyhouse, all morphological parameters and biomass were recorded from randomly harvested individuals (n = 5) from each treatment. For biomass estimation the cuttings were oven dried at 60°C for 72 hours and then dry weights were measured. The absolute growth rate (AGR) for plant height, leaf area, leaf number and biomass were estimated using following relation (1):

$$\text{AGR} = \frac{MT2 - MT1}{T2 - T1} \qquad (1)$$

Where: MT2 = value of morphological parameter on time T2 and MT1= value of morphological parameter on time T1.

AGR showed increased in morphological/biomass parameter in cm/g per day.

Data analysis To test the significant effect of PGR's treatment on morphology and biomass one way Analysis of Variance (ANOVA) was analyzed.

The significant difference at each treatment level was further analyzed by Duncan Multiple Range Test (DMRT) using IBM SPSS (Version 22.0).

Results

The effect of PGR's treatment on morphological parameters was found time dependent, the individuals from some PGR's was not survived when they transplanted to their natural habitat. The auxins like NAA in higher concentration (1000µM) and IAA in lower concentration (50 and 100µM) were unable to protect the cuttings from transplantation stress in alpine condition. The higher concentrations of cytokinin (BAP) also found ineffective once the plants transplanted to natural habitat.

The plant height and leaf area were found greater in IBA when applied in low concentration and the effect was consistent till 300DAP, however the growth rate for plant height and leaf area was found higher under IBA 100µM. The LN was found greater in NAA 100µM at 150 DAP but from 150 to 300days the leaf number multiplication was found higher in IBA 100µM, which was two folds higher than control **(**Table 1).

Table 1. Estimation of morphological characteristic of plants raised through shoot cuttings of *N. grandiflora*. PH, LA, LN, RN, RL, RD, DW. Similar alphabets in the column showed non-significant difference

Treatments	PH			LA			LN		
	150 DAP	300 DAP	AGR	150 DAP	300 DAP	AGR	150 DAP	300 DAP	AGR
Control	18.17±3.41cde	21.47±1.56cde	0.02	23.05±6.29e	27.27±2.66de	0.03	8.67±1.15abc	13.67±0.67a	0.03
NAA50	14.37±4.01bcd	17.47±1.54abc	0.02	15.21±5.74bcd	20.8±1.88bc	0.04	16.33±2.08def	21.33±1.45bcd	0.03
NAA100	16.1±1.44bcde	18.8±1.46abcd	0.02	18.25±1.72cde	21.47±1.58bcd	0.02	19±4.58f	24±1.53d	0.03
NAA 500	17.67±4.19cde	21.03±1.64cde	0.02	19.28±5.6cde	22.3±3.27bcd	0.02	10±4abcd	16.33±1.2ab	0.04
NAA 1000	14.67±2.08bcd	0	0	12.77±1.94bc	0	0	6.33±1.15ab	0	0
IBA50	20.8±2.17e	24.53±1.16e	0.02	24.3±5.16e	27.27±2.66de	0.03	14.33±2.31cdef	21±2.08bcd	0.04
IBA100	12.73±4.15bc	17.97±1.28abcd	0.03	12.83±4.37bc	20.8±1.88bc	0.04	17.33±6.51ef	26±1.15d	0.06
IBA500	14.87±0.32bcd	17±0.96abc	0.01	12.95±2.91bc	21.47±1.58bcd	0.03	15±3.61cdef	23.33±2.33d	0.06
IBA1000	11.07±2.69ab	14.47±1.05a	0.02	8.3±2.94ab	22.3±3.27bcd	0.04	7.67±1.53ab	15.67±1.2a	0.05
IAA50	16.3±1.97bcde	0	0	19.17±4.85cde	0	0	6.67±1.15	0	0
IAA100	7.47±0.9a	0	0	4.6±1.48a	0	0	5±2.65a	0	0
IAA500	19.5±5.27de	22.37±2.22de	0.02	21.69±6.52de	26.67±1.92cde	0.03	12±5.29bcde	17.67±1.76abc	0.04
IAA1000	14.3±0.3bcd	17.67±0.8abc	0.02	14.77±1.99bcd	18.8±1.19ab	0.03	14.67±3.79cdef	22.67±2.19cd	0.05
BAP50	13.4±0.85bc	15.43±0.5ab	0.01	13.22±0.63bc	16.37±0.49ab	0.02	10.67±1.53abcd	15±1.73a	0.03
BAP100	11.1±2.29ab	0	0	9.77±3.14ab	0	0	6.33±1.15ab	0	0
BAP500	10.83±2.25ab	0	0	8.34±3.09ab	0	0	13±3bcdef	0	0
BAP1000	18.07±3.96cde	0	0	18.5±2.7cde	0	0	12.67±5.69bcdef	0	0
BAVASTINE	16.23±1.62bcde	0	0	15.36±1.61bcd	0	0	8.67±2.89abc	0	0

The underground parts of the plant were also affected by PGR's treatment, the RN was found higher in IBA 50, the effect was non-significantly different from control at 150DAP but at 300DAP it was 28% higher than control; however the RL was significantly higher in control at both 150 and 300DAP. The PGR IBA in 50 and 100µM concentrations showed faster growth in RL in comparison to other PGRs and control. The RD at 150DAP was found higher for control but later it was found rapidly increased in IBA 50µM followed by IAA 500µM. The biomass in IBA 50µMwas found 49% higher than control at 150DAP and 53% higher at 300DAP. In IBA 50µM the per day dry weight accumulation was 6.1mg/day which is significantly greater than other treatment and control (3.8mg/day). The other PGRs treatments like NAA 100 and 500µM also accumulates significant amount of biomass but the difference was non-significant than control (Table 2).

Table 2. Effect of different PGRs on underground parts and Biomass of *N. grandiflora.* The similar alphabets in the column showed non-significant difference

Treatments	RN		RL		RD		DW (gm)		AGR (mg/day)
	150 DAP	300 DAP	150 DAP	300 DAP	150 DAP	300 DAP	150 DAP	300 DAP	
Control	15.67±4.16cdef	19.67±1.86ab	7.77±2.82d	13.1±1.3e	1.3±0.12e	1.69±0.16bc	1.1±0.45bcdefg	1.67±0.2cde	3.80
NAA50	9.33±2.52ab	16.33±1.45ab	4.17±0.76c	9.27±0.97cd	0.94±0.37cde	1.38±0.19abc	1.22±0.17cdef	1.38±0.08abc	1.04
NAA100	15±1bcdef	20±1.15ab	3.33±1.04abc	7.53±0.44abc	0.41±0.07abc	0.95±0.05ab	1.47±0.37abcdef	1.83±0.16de	2.40
NAA 500	14±2.65bcdef	17.67±1.67ab	3.83±0.7bc	7.63±0.49abc	1.07±0.69de	1.57±0.43abc	1.04±0.54bcdefg	1.86±0.04de	5.47
NAA 1000	13.67±1.53bcdef	0	2.53±0.12 abc	0	0.71±0.54abcd	0	0.47±0.15ab	0	0
IBA50	18.33±2.65ef	25.33±1.76c	4.07±1.01c	10.7±0.47d	0.87±0.09bcde	1.92±0.11c	1.64±0.36g	2.56±0.2f	6.13
IBA100	15.33±2.52bcdef	16.67±1.86ab	3.43±0.93 abc	9.57±0.5cd	0.7±0.44abcd	1.58±0.59abc	0.8±0.18bcde	1.4±0.09abc	3.96
IBA500	14.67±2.52bcdef	20.33±2.03b	3.27±0.21 abc	8.8±1.08bcd	0.62±0.16abcd	1.2±0.13abc	1±0.16bcdefg	1.46±0.11bcd	3.04
IBA1000	10.33±2.08abcd	16±0.58ab	2.3±0.92abc	6.37±0.49ab	0.31±0.1ab	0.9±0.03ab	0.51±0.03def	1.3±0.08abc	5.29
IAA50	13.33±3.06bcdef	0	1.93±0.49ab	0	0.55±0.15abcd	0	0.84±0.13abcdef	0	0
IAA100	4.67±2.52a	0	1.57±0.51a	0	0.25±0.08a	0	0.86±0.52abcdef	0	0
IAA500	16.33±4.04def	20.33±1.2b	3.43±0.35 abc	6.6±0.95ab	0.8±0.02abcde	1.62±0.07abc	1.3±0.49efg	1.72±0.16cde	2.76
IAA1000	11.67±1.53bcde	17.33±0.88ab	2.53±0.5 abc	5.6±0.44a	0.53±0.25abcd	1.01±0.03ab	0.64±0.21abc	0.98±0.06a	2.31
BAP50	14.33±1.53bcdef	16±1.15ab	3.13±0.55 abc	7.13±0.55abc	0.64±0.33abcd	1.13±0.14ab	0.77±0.24bcd	1.01±0.14a	1.56
BAP100	9.67±3.51abc	0	1.9±0.1ab	0	0.33±0.1ab	0	0.35±0.06a	0	0
BAP500	16.67±4.16def	0	3.07±1.29 abc	0	0.3±0.04a	0	0.63±0.16	0	0
BAP1000	18±7.64f	0	3.33±0.76 abc	0	0.47±0.29abc	0	1.43±0.69fg	0	0
BAVASTINE	16.33±2.08def	0	3.5±1.32 abc	0	0.74±0.1abcd	0	1.08±0.04bcdefg	0	0

Figure 1. (A) Well established cuttings of *N. grandiflora*, (B) Uprooting of cuttings from tray for transplantation to natural habitat, (C-D) Preparation of field for transplantation, (E) Transplanted seedlings into raised beds, (F) Mature uprooted cuttings after 300DAP

Discussion

Plant cultivation is directly depends on the rate of survival of transplanted plants, treatments, age and type of propagation parts, soil moisture and pH, nutrients availability and environmental factors. Hence cultivation of this species can be done from vegetative means of propagation compared to seeds because of low seedling survival even after good seed germination this is also one of the major factors for declining of this species in its natural habitat. Larger plants size with more number of roots has better ability to tolerate stress when transplanted in alpine areas compared to those of small size. Thus, cuttings of 150DAP selected for better survival at 3400m asl. [5]**.** Auxins are well known major group of hormones (IBA, IAA, NAA), which initiates sprouting of new leaves and root formation in cuttings and commonly used by means of vegetative propagation of plants**.** Since most of the cuttings treated with BAP showed no result with relation to rooting it may be due to the inhibitory capacity of this hormone since it affects the rate of uptake by tissues, to the target tissue from where roots are formed with respect to other PGR's. Concentration of cytokinin's may highly affect the role of auxins, when applied at higher concentration it reduces the rooting, while it enhances the rooting at lower concentration and hence considered as auxin-antagonists [15]. Results show that BAP treated cuttings did not show any responses after 300

DAP and all cuttings were dead. Combination of IBA and NAA treatments raised the number of roots in *Lilium martagon* L. as compared to other hormones [14, 16]. Basically, carbohydrate does not initiate rooting, but acts as a source of energy for the synthesis of root formation and other vital activities of plants. Here the experiment revealed that top edge cuttings which were treated with IBA 50µM shows highest survival (60%) rooting (70%) and maximum biomass compared to other treatments, because of its higher constancy, transportability, capability to produce roots and accordingly results in lower morality in plants. Since for proper growth of roots there are many factors which effect the survival of cuttings, initiation of new roots which is influenced by many factors like-age of selected plant, source of mother plants, rooting media, environmental factors and physiological status of mother plants [17-21]**.** Since the survivability of the investigated plant was higher during 150DAP but after 300DAP the survivability percentage get reduced to 60% due to both biotic and a biotic factors

like changes in weather pattern in alpine as compared to the investigated area where experiment was plotted.

There are several researchers also reported that application of auxin treatments mostly IBA and IAA enhanced root initiation as well as root number in many species [22-24]**.**

The increased number of roots per cutting resulted in reduced average length of roots [25]. For better establishment of those plants showed better results that have a greater number of roots rather than on the basis of root length [26]**.** Similar results were also found with respect to lower concentration of IBA as they induce positively all root parameters and biomass in *Angelica glauca* and *Heracleum candicans* [27-28]. In some medicinal and aromatic plants of high alpines (3000-3300m asl) like *Angelica glauca* and *Angelica archangelica* when experiments of cuttings were done the results clearly interpret that at minimum level of concentration were found to be best in IAA, IBA and GA3 [29-31] and suggested that low concentration of IBA (0.1%) showed significantly better rooting result and survivability compared to control in *Litsea monopetala*. In this study the overall growth and development of cuttings was better with IBA 50µM than that of NAA, IAA and BAP and required low concentration of PGR's.

Conclusion

From the last few decades, the conservation status of *N. grandiflora* is towards a declining manner it's due to unscientific overexploitation of this aromatic plant species for traditional and medicinal uses. But the cost of this all burden is coming over the Mother Nature. Since the main reason of overexploitation of this plant species is the superior aromatic fragrance of rhizomes that's why it's being continuously uprooted from the wild which is ultimately hampering the rate of reproductive phase, since in this plant the flowering occurs during the onset of monsoon period but as if people harvest the whole or part of that plants at this peak flowering time it will directly affects the seed setting ratio. For the fast multiplication, sustainable use and conservation point of view, the fully mature shoots used as mass propagating material were found quite beneficial in present study. In present study, it has also proved that the use of fully mature shoots of *N. grandiflora* gives instant growth measures as compared to conventional seed method. The growers/cultivators may be suggested that they can use the above ground part i.e. fully mature shoot as an extra planting material for their fields, since it will be dry and died (shoot senescence) during the winter season without any use. If these shoots treated with the lower concentration of auxins (IBA) helped in more root formation followed by good establishment of the plants in field. Simultaneously, the remaining underground parts i.e., rhizomes of the plants can be used for further cultivation practices and also take off for traditional and pharmaceutical uses.

Abbreviations

DAP = Days after planting. **IBA** = Indole-3-butyric acid, **IAA** = Indole-3-acetic acid, **NAA** = Naphthalene acetic acid, **BAP** = Benzylaminopurine, **µM** = Micro mole, **FYM** = Farmyard manure, **FL** = Forest litter, **PGR's** = Plant growth regulators.

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