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Effective Regeneration of *Impatiens Campanulata* Wight a Valuable Medicinal Plant from Western Ghats of Tamil Nadu, India

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Abstract: An in vitro plant regeneration protocol was successfully established in *Impatiens campanulata*, an important medicinal plant by tissue culture methods.The plants were raised and development of protocorm was highest in MSmedium. In the present investigation universal media MS, B_5 and WPM were used. The composition of three different media is represented are very active for regeneration of *Impatiens campanulata*.The principal component of most tissue culture media can be categorized into inorganic nutrients (macro and micro), organic nutrients (vitamins and amino acids), carbon source, plant growth regulators and gelling agent. . To standardize a perfect protocol for *In vitro* rooting and subsequent acclimatization of plantlets of *Impatiens campanulata.*

Key words: *Impatiens campanulata* · Tissue culture · Inorganic nutrients · Regeneration

INTRODUCTION

Today over 95% of the medicinal plants used by the industries in India are collected from forests and other natural resources. Less than 20 plant species are collected under commercial cultivation, while over 400 species used for industrial production are from wild population. Over 70% of the plant collection involves destructive harvesting because of the use of parts like bark, root, stem, seed and whole plant. In south India alone, it is estimated that about 100 out of 300 species are either severely endangered or threatened [1].

The World Health Organization (WHO) estimated that 80% of the population of developing countries still relies on traditional medicines, mostly plant drugs for their primary health care needs. Now day's medicinal plants constitute a group of industrially important crops which bring appreciable income to the country and by way of valuable foreign exchange. Medicinal plants have become a never ending source for new-bio dynamic compounds of potential therapeutic values. Plant based drugs represent a stable market upon which both physicians and patients seem to rely. Many drug companies, taking clue from traditional system, have started actively researching to bring the use of plant extractions in several preventive and curative medicines aimed at new drug discoveries for cancers, cardiovascular and other diseases.

Due to fast changing life style globally today the demand for plant based pharmaceuticals is continuously on the rise. Though medicinal plants are of great commercial value most of them are still of wild origin. Because of increase in demand the ruthless collection of these plants from natural sources have resulted in their rapid depletion.

Endemicity, restricted distribution, small population, inaccessible areas and anthropogenic pressure have caused a decline in wild population of many species making their status rare[2]. Continuous and unorganised exploitation has resulted in many plants becoming rare and some even becoming extinct [3]. Biopiracy of rare and endangered plants is also threat to their survival [4].

So *in vitro* regeneration is an efficient means of *ex situ* conservation of plant diversity because with this technology many endangered species can be quickly propagated and preserved from a minimum of plant material and with little impact on wild population. Moreover this technique has the unique advantage of propagation of the desired taxon, independent of season, reproductive barriers, germination hurdles and so on [5]. In fact *In vitro* propagation and cryopreservation of

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medicinal plants help us to conserve biodiversity. Cryopreservation is a reliable method for long-term storage of the germplasm of endangered species.

Cultivation of medicinal plants is also difficult due to lack of standardized agronomic practices for the most species and unavailability of source plant material. An alternate method is the micropropagation, which can produce large-scale plantlets within short time.

In recent years biotechnology has emerged as a frontier branch of science increasingly being used in several areas. Biotechnology, a great revolutionary technique of the century, offers solution to many problems in medicine, health care and conservation. Tissue culture is one of the techniques in Biotechnology which has brought about significant impact in the field of plant breeding and conservation of many endangered plants. Today, the unique regeneration properties of plants and their biochemical potential have been exploited fully. Biotechnological approaches are being employed to the production of secondary metabolites for pharmaceutical use.

Impatiens have a broad distribution and can be found growing throughout tropical Africa, India, southwest Asia, southern China, Japan, as well as parts of Europe, Russia and North America [6]. *Impatiens* and *Hydrocera* are the sole genera that comprise the family Balsaminaceae. The genera *Impatiens* is estimated to contain between 400-850 species [6] world wide and about 120 species were reported in India. *Impatiens,* primarily annual or perennial herbs, can occasionally become subshrub. Generally, they have thick, fleshy stems, with thin fleshy leaves that become membranous and almost transparent when dried. Similarly, the flowers are fleshy and often display a characteristic sheen caused by light reflected from the surface cells. *Impatiens* flowers are short-lived and when pollinated produce fruit with abundant seeds which are expelled forcefully from .explosive fruits.

The present investigation was undertaken with the following objectives. To standardize a perfect protocol for *In vitro* rooting and subsequent acclimatization of plantlets of *Impatiens campanulata.*

MATERIALS AND METHODS

Plant Material: Impatiens campanulata Wight was collected from Palani hills near Kodaikanal in Tamilnadu. The plants were potted in pots and maintained at greenhouse conditions. The plants were grown at 30°-40°C with water as required.

Media Preparation and Sterilization: Nutritional requirements for optimal growth on a tissue *in vitro* may vary with the species. Even tissues from different parts of a plant may have different requirements for satisfactory growth [7] There is no single medium that is suitable for all types of plant tissues and organs. So, for convenience, three media that represent high, medium and low salt concentrations were selected for present investigation.

In the present investigation universal media MS [7], B_5 [9] and WPM [10] were used. The composition of three different media is represented in Table 1. The principal component of most tissue culture media can be categorized into inorganic nutrients (macro and micro), organic nutrients (vitamins and amino acids), carbon source, plant growth regulators and gelling agent. Other organic supplements such as CH and CM were also added to the medium when required. All the chemicals used in media preparation were of analytical grade. The hormones were Sigma make. Bacteriological grade agar was used as gelling agent throughout the study. Natural daylight and irrigated with water as required.

In Vitro Rooting: Microshoots with 4-5 nodes were cultured on different media such as full strength, half strength and quarter strength MS, B_5 and WPM media supplemented with 1% sucrose, 0.6% agar and auxins. After selecting the best basal medium, the medium is supplemented with different concentrations of auxins.

Acclimatization: *In vitro* raised plantlets with well developed rooted shoots were taken out of the test tubes and washed with sterilized double distilled water to remove all the traces of agar. Then these plantlets were planted in earthen ware pots containing sterilized sand, farmyard manure and peat moss in 1:1:1 ratio, covered with big polythene bags and incubated at 25±2°C for 20 days. During this period pots were irrigated with liquid half strength MS basal nutrient medium without sucrose. Later small perforations were made on the polythene bag to reduce the relative humidity. Slowly the width of the holes was increased until the relative humidity inside the polythene bag and outside the chamber come to equal. After this conformity polythene bag was removed and the pots were directly exposed to the controlled temperature (25±2°C). Slowly the pots were transferred to room temperature having diffused light and plants were shifted to the green house and finally plants were transferred to the Sunlight.

Table 1: Effect of various concentrations of BAP, KN, 2-iP and Zeatin on multiple shoot induction from mature nodal explant of *I. campanulata* cultured on MS medium

Plant growth regulators (mg/l)		Shoot sprouting frequency (%)	Shoot No. per explant Mean±SE	Shoot length (cm) per explant Mean±SE
BAP	0.1	0	NR	NR
	1.0	70	1.61±0.04^{bc}	1.10±0.02^{d}
	2.0	85	2.09±0.02^{a}	2.00±0.03^{ab}
	3.0	60	1.84±0.01^{b}	1.48±0.02^{b}
	5.0	55	1.56±0.02^{c}	1.28±0.02^{c}
	8.0	40	1.32±0.03^{cd}	1.21±0.05^{cd}
KN	0.1	50	1.00±0.00^{e}	1.00±0.02^{de}
	1.0	60	1.51±0.01^{bc}	1.36±0.03^{bc}
	2.0	55	1.29±0.02^{cd}	1.18±0.02^{cd}
	3.0	45	1.20±0.02^{d}	1.10±0.03^{d}
	5.0	40	1.17±0.01^{d}	0.92±0.02^{e}
	8.0	0	NR	NR
2-ip	0.1	0	NR	NR
	1.0	75	1.92±0.02^{ab}	2.18±0.04^{a}
	2.0	68	1.60±0.01^{bc}	1.46±0.02^{b}
	3.0	60	1.43±0.01^{c}	1.30±0.03^{bc}
	5.0	45	1.22±0.02^{d}	1.20±0.02^{cd}
	8.0	40	1.14±0.01^{de}	1.18±0.03^{cd}
Zeatin	0.1	45	1.18±0.02^{d}	0.97±0.09^{e}
	1.0	68	1.60±0.03^{bc}	1.32±0.03^{bc}
	2.0	55	1.30±0.02^{cd}	1.26±0.01^{c}
	3.0	50	1.21±0.07^{d}	1.22±0.04^{c}
	5.0	48	1.15±0.09^{d}	1.18±0.02^{cd}
	8.0	40	1.12±0.07^{de}	1.10±0.02^{d}

Values represent mean±standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

Statistical Analysis: Each culture tube with one shoot explants was considered as one replicate. Each treatment in each set of experiments consists of 15 replicates and each experiment was repeated three times. Standard error of means was calculated in each experiment. The data was statistically analyzed using one way analysis of variance (ANOVA) and means were compared using the Tukey test at the 0.05% level of significance.

RESULTS AND DISCUSSION

In combination with BAP + Zeatin, BAP 2.0 mg/l + Zeatin 1.0 mg/l gave maximum number of 2.25±0.01 shoots /explants (Table 1) than BAP 1.0 mg/l + Zeatin 1.0 mg/l (1.80±0.01 shoots / explant). Combination of 2-ip and Zeatin showed very least number of shoots among BAP + 2-ip, BAP + KN and BAP + Zeatin.

Among various combinations of 2-ip and Zeatin maximum shoot number of 1.70±0.02 shoots / explant with maximum shoot length 1.25±0.02 cm was observed on 2-ip 1.0 mg/l + Zeatin 1.0 mg/l (Table 1). Of the various combinations of BAP + 2-ip + Zeatin tested, medium supplemented with BAP 2.0 mg/l + 2-ip 1.0 mg/l + Zeatin 1.0 mg/l only produced maximum induction of 1.84±0.01 shoots / explant with 2.04±0.02 cm of shoot length (Table 2).

In our present investigation it was found that MS medium containing BAP+ Zeatin, 2-ip+Zeatin and BAP+2-ip+Zeatin was less effective for shoot multiplication compared to BAP + 2-ip which gave maximum number of shoots per explant in *I. campanulata.*

The synergistic effect of growth regulators on promotion of shoot multiplication was well documented for many plant species. Various successful combinations have been reported by Biggs [10] and Hossain [11] Media containing combination of different cytokinins may increase the number of shoots formed when compared to media with only one cytokinin.

Effect of Cytokinin and Auxin Combination for Shoot Multiplication: In a number of cases cytokinin alone is enough for optimal shoot multiplication. However axillary shoot proliferation in some species may be promoted by the presence of an auxin and cytokinin.

Table 2: Effect of various combinations of BAP, 2-iP and Zeatin on shoot induction from nodal segments of *I. campanulata* on MS medium

Plant growth regulators (mg/l)			Shoot sprouting	No. of shoots/	Shoot length (cm)
BAP	2-iP	Zeatin	frequency (%)	explant Mean±SE	Mean±SE
1.0	-	0.1	50	1.05±0.01^{g}	1.02±0.01^{f}
1.0	-	0.5	60	1.20±0.01^{e}	1.10±0.01^{ef}
1.0	-	1.0	70	1.80±0.01^{b}	1.30±0.01^{d}
1.0	-	2.0	45	1.32±0.01^{e}	1.15±0.02^{e}
2.0	-	0.1	55	1.16±0.02^{f}	1.28±0.01^{d}
2.0	-	0.5	68	1.35±0.01^{e}	1.40±0.01^{cd}
2.0	-	1.0	75	2.25±0.01^{a}	1.95±0.02^{ab}
2.0	-	2.0	60	1.45±0.01^{de}	1.45±0.02^{cd}
3.0	-	0.1	52	1.13±0.03^{f}	1.25±0.02^{d}
3.0	-	0.5	55	1.25±0.01^{ef}	1.30±0.01^{d}
3.0	-	1.0	65	2.12±0.01^{ab}	1.50±0.02^{cd}
3.0	-	2.0	50	1.30±0.01^{e}	1.28±0.02^{d}
-	1.0	0.1	30	1.14±0.02^{f}	1.08±0.02^{ef}
-	1.0	0.5	45	1.24±0.02^{ef}	1.10±0.03^{ef}
-	1.0	1.0	65	1.70±0.02^{c}	1.25±0.02^{d}
-	1.0	2.0	55	1.28±0.03^{ef}	1.16±0.02^{e}
-	2.0	0.1	25	1.09±0.02^{fg}	1.00±0.01^{g}
-	2.0	0.5	30	1.25±0.01^{ef}	1.06±0.01^{ef}
-	2.0	1.0	60	1.60±0.01^{cd}	1.20±0.02^{de}
-	2.0	2.0	50	1.23±0.02^{ef}	1.10±0.02^{ef}
2.0	1.0	1.0	65	1.84±0.01^{b}	2.04±0.02^{d}
2.0	2.0	2.0	50	1.55±0.03^{d}	1.60±0.02^{b}

Values represent mean±standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

Table 3: Effect of different conentrations of BAP in combination with different concentrations of IBA on shoot proliferation from mature nodal explants of *I. campanulata*

Plant growth regulators (mg/l)				
BAP	IBA	Shoot sprouting frequency (%)	Shoot no. per explant Mean±SE	Shoot length (cm) Mean±SE
1.0	0.1	50	1.36±0.02^{e}	1.26±0.03^{f}
1.0	0.5	72	1.82±0.01^{c}	2.43±0.02^{b}
1.0	1.0	60	1.57±0.01^{d}	1.48±0.02^{e}
1.0	2.0	55	1.20±0.01^{f}	1.25±0.02^{f}
2.0	0.1	65	2.53±0.01^{b}	2.08±0.02^{bc}
2.0	0.5	80	3.82±0.02^{a}	3.10±0.02^{a}
2.0	1.0	73	1.81±0.01^{c}	1.76±0.01^{c}
2.0	2.0	68	1.44±0.04^{de}	1.38±0.03^{ef}
3.0	0.1	60	1.54±0.02^{d}	1.34±0.01^{ef}
3.0	0.5	75	1.79±0.02^{cd}	1.60±0.02^{d}
3.0	1.0	65	1.46±0.03^{de}	1.46±0.01^{e}
3.0	2.0	60	1.26±0.03^{f}	1.20±0.01^{f}

Values represent Mean±SE, of 15 replicates per treatment in three repeated experiments. Mean followed by the same letter are not significantly different by the Tukey test at 0.05% probability level

Table 4: Effect of different conentrations of BAP in combination with different concentrations of 2,4-D on shoot proliferntion from mature nodal explants of *I. campanulata*

Plant growth regulator (mg/l)				
BAP	2,4-D	Shoot sprouting frequency (%)	Shoot no. per explant Mean±SE	Shoot length (cm) Mean±SE
1.0	0.1	45	1.21±0.02^{de}	1.17±0.02^{de}
1.0	0.5	55	1.62±0.01^{b}	1.30±0.01^{c}
1.0	1.0	38	1.50±0.02^{bc}	1.20±0.02^{d}
1.0	2.0	25	1.15±0.02^{e}	1.08±0.02^{e}
2.0	0.1	50	1.30±0.02^{d}	1.48±z.02^{b}
2.0	0.5	65	1.95±0.02^{a}	1.82±0.01^{a}
2.0	1.0	55	1.42±0.02^{c}	1.50±0.01^{b}
2.0	2.0	30	1.22±0.01^{d}	1.32±0.01^{c}
3.0	0.1	48	1.01±0.01^{f}	1.10±0.02^{e}
3.0	0.5	56	1.50±0.01^{bc}	1.30±0.02^{c}
3.0	1.0	35	1.22±0.01^{de}	1.15±0.02^{de}
3.0	2.0	20	1.14±0.02^{e}	1.08±0.02^{e}

Values represent Mean±SE, of 15 replicates per treatment in three repeated experiments. Mean followed by the same letter are not significantly different by the Tukey test at 0.05% probability level

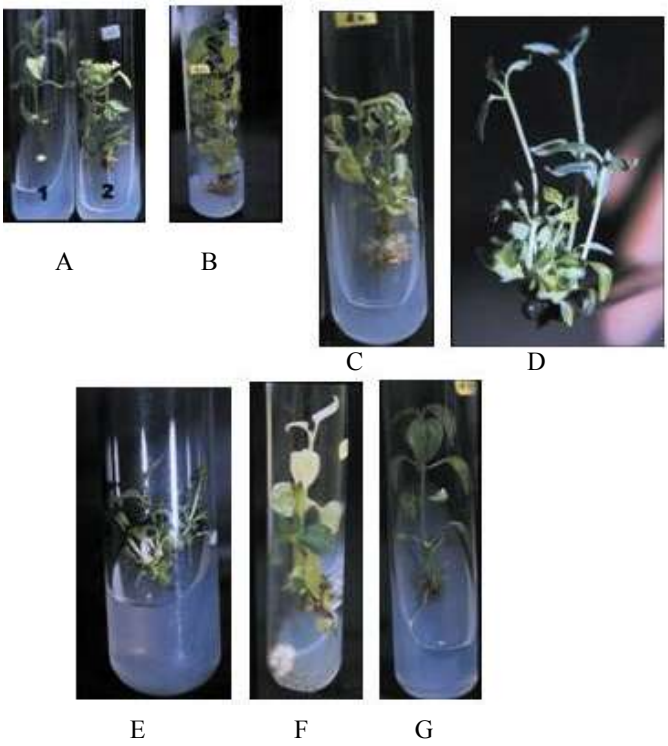

Fig. 1: A-C-Microshoots cultured on ½ MS + 0.1 mg/l NAA 1 D-G-Microshoots cultured on ½ MS + 2 mg/l NAA, 3mg/l NAA and 5.0 mg/l NAA respectively

Assuming that combined effect of auxin and cytokinin could improve further multiplication rate of shoots, different concentrations and combinations were studied.

Interaction between the classes of regulants are often complex and more than one combination of substances is likely to produce optimum results. Hence, in the present study different concentrations of BAP in combination with different auxins such as IBA and 2,4-D were used on MS medium for optimizing multiple shoot regeneration (Table 3-4).

Table 5: Effect of various auxins on rooting response from *in vitro* regenerated shoots of *I. campanulata* cultured on MS half strength medium after 30 days

Concentration of Auxin mg/l					
NAA	IAA	IBA	% of response	Number of Roots/ shoot Mean±SE	Length of roots (cm) Mean±SE
0.10	-	-	75	10.37±0.05^{a}	3.61±0.02^{ab}
0.50	-	-	65	6.24±0.02^{bc}	2.12±0.02^{b}
1.00	-	-	50	4.19±0.03^{cd}	1.42±0.02^{cd}
2.00	-	-	-	CP	CP
3.00	-	-	-	CP	CP
-	0.10	-	60	5.25±0.04^{c}	1.50±0.02^{c}
-	0.50	-	70	7.12±0.02^{b}	2.48±0.02^{b}
-	1.00	-	55	3.25±0.03^{d}	1.40±0.02^{cd}
-	2.00	-	50	2.16±0.02^{e}	1.22±0.02^{d}
-	3.00	-	-	CP	CP
-	-	0.10	45	3.19±0.03^{d}	2.24±0.03^{b}
-	-	0.50	68	5.28±0.03^{c}	4.09±0.02^{a}
-	-	1.00	60	2.19±0.03^{e}	1.24±0.02^{d}
-	-	2.00	-	CP	CP
-	-	3.00	-	CP	CP

Values represent mean±standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

Among various combinations of BAP + IBA best response in terms of multiple shoot regeneration was observed on MS medium supplemented with BAP 2.0mg/l + IBA 0.5 mg/l with an average of 3.82± 0.02 shoots/ explant (Fig.1A) (Table 6) from nodal explant of *I. campanulata*. Out of three concentrations of BAP + 2, 4-D combinations tested BAP 2.0 mg/l + 2, 4-D 0.5 mg/l produced maximum of 1.95±0.02 shoots/ explant and shoot length 1.82±0.01cm (Table 4) (Fig.1B-G). Further increase in the 2, 4-D concentration decreased the shoot number.

With regard to plant growth regulators, in micropropagation the general principle is that during *in vitro* culture, presence of cytokinin (with out addition of auxin) in the medium promotes the induction of shoot morphogenesis. However in the present study *I. campanulata* addition of auxin to the cytokinin enriched medium enhanced the shoot number, but also caused the growth of unnecessary basal callus.

Among different combinations of BAP and auxins tested for shoot regeneration IBA gave maximum number of shoots when compared to other auxins and the order of response is IBA>2,4-D. Shoot number and shoot sprouting frequency with cytokinin and auxin treatment was much satisfactory when compared to cytokinin combinations.

Improvement in shoot regeneration with cytokinin + auxin combination was proved in several species such as *Trichopus zeylanicus* [12], *Decalepis hamiltonii* [6], *Enicostemma hyssopifolium* [13], *Hedychium spicatum* [14], *Ocimum gratisimum* [15], *Salvadora persica* [16] and *Smilax zeylanica* [17].

***In vitro* Rooting and Acclimatization:** After *In vitro* regeneration of plantlet the important aspect in tissue culture is rooting and acclimatization. Efficient rooting of *In vitro* regenerated plants and subsequent field establishment in the last and crucial stage of rapid clonal propagation. *In vitro* rooting was practically the only method used for obtaining plantlets [18]. *In vitro* rooting was essential, if the shoots are very sensitive to drought [19]. In such instances, the extra cost of an *in vitro* rooting stage may be justified if it results in plantlets of better final quality (or) if losses during the acclimatization stage can be reduced. Some herbaceous species root more readily and survive better and have a higher growth rate and produce more auxiliary shoots if they are rooted *in vitro* rather than directly rooted *ex vitro*.

Out of the three media used for root induction MS medium was found to be superior over B_5 and WPM. Out of the two types of MS media used MS with half strength was superior over MS full strength in both the taxa. Concentration of mineral salts in the medium play an important role in root induction. High salt levels are frequently inhibitory to root initiation [20]. Corresponding with the report FOR *Eremostachys superba* [21], ½ strength MS medium was found to be better than full

strength MS medium. In the present investigation ½ strength MS medium with various concentrations was optimum for root induction. ½ strength MS medium produced 10.37±0.03 roots with 75% of response where as MS medium produced 6.51±0.05 roots with 65% response.

Effect of Auxins on *In vitro* Rooting of *In vitro* Derived Shoots: Generally treatment with growth regulators considerably speeds the process of rooting to produce more roots. *In vitro* shoots were rooted maximum on ½ strength MS medium, when compared to full strength MS medium. Half strength MS medium supplemented with three auxins such as NAA, IAA and IBA at different concentrations (0.1-3.0 mg/l) showed varied effect of rooting (Table 5).

Auxins played a vital role in root induction. Differences were noticed in the nature of roots induced depending on the auxin used in the medium. Out of the three auxins tested NAA was found to be effective for root induction when compared to other two auxins in both the plants.

For induction of rooting in *I. campanulata* NAA was found to be effective than IAA and IBA of all the concentrations tested NAA at 0.1 mg/l in the ½ strength MS medium proved to be the best. NAA 0.1 mg/l produced 10.37±0.03 roots (Plate 3A-C) with 75% of rooting with increase in the concentration of NAA up to 1.0 mg/l there is decrease in frequency of rooting and root length. Finally there is no root induction of NAA 2.0 mg/l and 3.0 mg/l (Table 8) (Fig.1D-G). IAA at 0.5 mg/l showed showed maximum of 70% response in 15 days with 7.12±0.02 roots/shoots with 2.48±0.02 cm length (Table 5). IBA at 0.5 mg/l showed maximum response of 68% in 10 days with 5.28±0.03 roots per shoot and attained about 4.09±0.02 cm of maximum shoot length.

The reason for the reduced survival in higher concentrations of auxin treatments may be due to poor vascular connection of the root with the stem because on the intervention of callus.

The number of roots formed on each shoot often increased in proportion to the concentration of auxin applied, but when the concentration becomes supra-optimal (2.0 mg/l) and callus formation is promoted and roots had an abnormal appearance and their average length and subsequent shoot growth decreased. The addition of high levels of IBA to the rooting medium sometimes caused shoot tip necrosis. However, it can be controlled by supplementing IAA in minute concentrations. So selection of an optimal intermediate level of auxin for rooting micro cutting is must.

Transferring of Plantlets to the External Environment: The ultimate success of *in vitro* propagation lies in the successful establishment of plants in the soil. Acclimatization of *In vitro* plantlets to green house (or) field condition is a critical step for many plant species requiring time and explants that restrict the commercial application of the micropropagation process. Prior to hardening plantlets rooted *in vitro* showed have well proportioned shoots and roots that are capable of supporting each other. The removal of the plantlets from *In vitro* conditions damaged the dedicated roots (or) even more likely they will have incomplete vascular system [22]. High humidity is essential for successful acclimatization. Usually most difficult step during micropropagation is the recovery of plants from the culture vessels into the soil.

The transplantation stage continues to be a major bottleneck in the micropropagation of plants. The benefit of any micropropagation system, can however, only be fully realized by successful transfer of plantlets from tissue culture vessels to ambient conditions found *ex vitro,* so, *in vitro* grown plantlets require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil.

Plantlets or shoots that have grown *in vitro* have been continuously exposed to unique microenvironment that has been selected to provide minimal stress and optimum condition for plant multiplication. Plantlets were developed within the culture vessels under low level of light aseptic conditions on medium containing ample sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity. One of the main reason for low acceleration rate *ex vitro* might be low photosynthetic rates *In vitro* presumably because of low photosynthetic photon flux (PPF) with a low CO_2 concentration in the cultural vessels are forced to open their stomata in order to maintain an equilibrium with the surrounding atmosphere . These conditions cause the improper development of stomata even if they are subjected to sudden environmental changes [23]. *In vitro* cultivated plants lack the necessary anatomical features to withstand variations in the natural environment [24]. Micropropagated plantlets cannot survive the environmental conditions when directly placed in a green house or field. Decreasing the water potential of the medium and reducing the humidity in the culture vessel can be achieved *In vitro* hardening of micropropagated plantlets. Maintaining plant in light of high irradiance before, during or after *in vitro* rooting facilitates establishment of plantlets.

Well rooted plantlets from the culture vessels were gently removed keeping the roots intact. The plantlets were transferred to a container of warm water and gently rinsed the agar-media off the roots and the regenerates were planted in a plastic cups with 1:1:1 (soil: sand: manure) sterile mix. The plants were wrapped in plastic paper to ensure high humidity and irrigated every two days with ½ strength MS liquid medium without any vitamins and sucrose. Lowering sucrose percent or omitting it altogether can give faster and more successful acclimatization [25]. The plantlets are maintained in the culture room for 15 days. After 15 days the wrap is removed and plants were allowed to adjust to ambient conditions. Then the hardened plantlets were transferred to earthen pots and maintained under shade for one week. Then plants were exposed to sun light for few hours for a week and then plants were transferred to soil and watered with tap water. The plantlets then performed all of their own photosynthesis and adapted to lower relative humidity by developing a waxy cuticle and regulating their function. The rooted plants were successfully established in soil with 75% survival rate.

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