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# Indirect Shoot Organogenesis from the Leaf Explants of *Oxalis corniculata* (L.) - An Important Medicinal Plant

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*Keywords:* Indirect **o**rganogenesis, Regeneration, Nodal and leaf Explants, *Oxalis corniculata*

## Abstract

Axillary bud explants of *Oxalis corniculta* grown in the field were excised and inoculated on MS medium with various concentrations and combinations of auxins (2, 4-D and NAA) and cytokinins (BAP and Kn) for indirect shoot formation through callus induction. Indirect organogenesis was achieved by the application of PGRs such as BAP, Kn, and NAA in the culture medium. The maximum mean shoot number (9.2) and regeneration frequency (77%) was found with BAP (2.0 mg/l) and NAA (0.5 mg/l). Healthy microshoots were separated and transferred to rooting medium supplemented with NAA, IBA and IAA. MS medium augmented with NAA 2.0 mg/l was found to be best for rooting. The regenerated plantlets were then hardened, acclimatized and where exhibited 75% survivability. The reproducible protocol can be used for regeneration and genetic transformation studies.

## Introduction

*Oxalis corniculata* Linn. is a medicinally important plant and it is known as creeping wood sorrel or sleeping beauty, resembles the common yellow wood sorrel. Although it is a naturally occurring weed, it has been used in traditional medicine for the cure of dysentery, diarrhea, and also it is a good source of vitamin C. In underdeveloped nations, more than 80% of the population relies only on herbal remedies for primary healthcare and the requirement of herbal medicines in developed nations is one-fourth of all prescribed medications come from wild medicinal plants (Chen et al. 2016, Kang et al. 2018). The world population is increasing year by year that brings changes in daily life

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like habitat destruction and wild populations of medicinal plants are being decreased (Chen et al. 2016). Micropropagation has been successfully utilised to increase plant populations in short duration than is possible with traditional propagation techniques (Das et al. 2020). *In vitro* propagation techniques serves in many aspects such as preservation of medicinal plants and generation of high quality explants for pharmaceutical industries (Nilanthi and Yang 2014, Komakech et al. 2020).

Micropropagation also provides a rapid and reliable system for production of a large number of genetically uniform disease free plantlets. It may be maximum beneficial for its mass propagation in addition to its conservation. Callus as such is highly required in transgenic studies to generate transformed plant cells from different types of explants. Callus also can be used to produce secondary metabolites and therapeutic antibodies (Efferth 2019). It can be subjected to either biotic or abioticelicitors which helps in enhancing desired secondary metabolites and also through metabolic engineering to improve therapeutic effects (Karwasara et al. 2010; Nandagopal et al. 2018; Efferth 2019). Taking into account the aforementioned information, the current study sought to develop sustainable conservation methods for *O. corniculata* and establish a viable protocol for indirect organogenesis using leaf and nodal explants with various combinations of growth regulators.

## Materials and Methods

The leaf and nodal explants were collected from the field grown *Oxalis corniculata* plants which were maintained in the herbal garden, Dravidian University, Kuppam, Andhra Pradesh, India. These explants were subjected to different sterilization agents like detergent solution 5% (v/v) Tween-20 or Teepol, 0.4% (w/v) bavistin, 70% ethanol and 0.1% mercuric chloride (Merck, India) for different time intervals and finally washed with sterile water. Explants were cut into small pieces of size 1.0-1.5 cm and transferred to MS medium supplemented with different combinations and concentrations of hormones such as Kn, BAP, NAA and IBA to produce callus and for the further development of shoots. All *in vitro* grown cultures were maintained under illumination of a 16 hrs photoperiod at 25 ± 2ºC. Fully elongated shoots were treated with different auxins such as NAA and IBA for induction of roots. After obtaining sufficient development of roots, plantlets were taken out and transplanted into small plastic bags containing sterile red soil and vermiculate at 1:1 (w/w). For mass propagation, the plantlets were transferred to large plastic pots for hardening. Finally plants were transferred to field.

All the experiments described above were conducted in a completely randomized design and repeated thrice and each treatment contains 10 replicates. Means and standard error of all dependent variables such as callus induction, shoot regeneration, shoot number and shoot length under different concentrations of plant growth

regulators were analyzed by ANOVA using SPSS software version 16.0 (IBM SPSS, IL) and mean was compared using DMRT at 5% significance.

## Results and Discussion

The present study was conducted to evaluate the effect of different auxins and cytokinins on the induction of calli and regeneration into whole plant from nodal and leaf explants of field grown plants of *Oxalis corniculata*. Young disease-free nodal and leaf explants of *Oxalis corniculata* from the two month old field grown plants were used for callus induction. MS gelled medium supplemented with different concentrations in singly and combination of auxins (NAA, IAA and 2, 4-D) and cytokinins (BAP and Kn).

For callus induction, nodal explants were collected from the two month old field grown plants. Nodal segments (0.5-1.0 cm length) were inoculated on MS medium augmented with different concentrations of auxins (NAA, IAA and 2, 4-D) in singly and combination with cytokinins (BAP and Kn) shows varied callusing response. The explants ineffective to form callus on MS medium without plant growth regulators but the swelling of explants is observed. Callus induction and proliferation was found in all the nodal explants of field grown plants (Fig. 1A-C). The callus initiated from the cut end portions of the explants where cells at the cut ends undergo mitosis which leads to callus formation and it is due to wound reaction of the explants or effect of exogenous growth hormones. The texture of callus differs based on the type of cytokinin and also on the auxin: cytokinin ratio (Martin 2002). In the present study, callus was stimulated from nodal segments were creamish green (Fig. 1A &B) and light green (Fig. 1C). 2, 4-D, and Kn in combination were observed to be effectual hormonal combination for callus induction from nodal explants. Callus was induced from nodal explants of *Centella asiatica* (Sekhar et al. 2014) by using MS medium with 2, 4- D alone and in combination with BAP. Callus was induced from nodal explants of *Centella asiatica* (Chandra sekhar et al. 2014) by using MS medium with 2, 4- D alone and in combination with BAP. Callus induction from nodal explants also reported in *Cissampelos pareira* where MS medium was supplemented with BAP, Kn either single or in combination with NAA 0.5 mg/l ( Reddy et al. 2019). It was well documented by Agastian et al. (2006) in *Justicia gendarussa* where chlorophyllus calli were formed from nodal bud explants using the combination of NAA and BAP.

The callus was induced after 4 weeks of inoculation in all the leaf explants of field grown plants (Fig. 1D-F). In the combination of 2, 4-D (0.5 mg/l) and BAP (0.5 mg/l) a green coloured callus was formed (Fig.1D). NAA (1.0 mg/l with BAP 0.5 mg/l), a compact white coloured callus was formed (Fig.1E). The green colour callus was further improved after increasing the concentration of 2, 4-D (1 mg/l) and without changing the concentration of BAP (0.5 mg/l) (Fig.1F). These findings are in line with earlier reports in *Justica gendarussa* (Agastian et al. 2006). It was also reported by Mehta et al. (2007) that

the callus induction from the leaf explants was observed on MS medium containing NAA and Kn. In several reports, NAA and BAP produced profuse, green compact callus. It was reported by Shasthree et al. (2009) that the combination of BAP and NAA shows nodular callus in *Erythrina variegata*.

Even though the callus production has been well optimized from both explants of nodal and leaf explants, the regeneration of whole plant is successful only with leaf explants and no regeneration was observed with nodal explants. Hence further experiments for whole plant regeneration are continued from the leaf explants. Plant propagation through callus requires the induction of organogenic callus. It was well documented by Harms et al. (1983) that the origin of explant source and its physiological state are vital factors for organogenic callus induction. Well developed calli derived from young leaf explants were sub-cultured on fresh MS medium augmented with different concentrations of BAP/Kn in alone or combination with auxin such as NAA. After two weeks of sub-culture, shoot buds were emerged from leaf derived callus (Table 1& Fig 2).

2, 4-D alone or in combination with cytokinins such as BAP and Kn is usually used to improve the callus induction and its maintenance (Castillo et al. 1998). There are many reports on shoot regeneration of various medicinal plants via callus cultures (Rao et al. 2005; Saritha et al. 2003). MS medium amended with BAP at low concentration, (0.5 mg/l) resulted in low frequency of shoot initiation (Fig. 2A). Further increase in BAP concentration, the shoot initiation frequency was increased (Fig. 2B). The microshoots were elongated at low level at MS medium with BAP (1.0 mg/l) + (0.5 mg/l NAA) (Fig. 2C) and improved well at MS medium with Kn (1.0 mg/l) + (0.5 mg/l NAA) (Fig. 2D). The maximum number of shoots (9.2 ± 0.5) was achieved in MS medium supplemented with BAP (2.0 mg/l) and NAA (0.5 mg/l) (Fig. 2E) and resulted 77% of regeneration frequency with decreased shoot length (2.9 ± 0.05). The maximum shoot length (6.1 ± 0.1) was achieved in MS medium supplemented with Kn (2.0 mg/l) and NAA (0.5 mg/l) with 76% regeneration frequency with multiple shoots (5.7 ± 0.1). The highest regeneration frequency 79% was achieved in MS medium supplemented with BAP (1mg/l) and NAA (0.5 mg/l) resulted multiple shoots (7.6 ± 0.1) with decreased shoot length (1.9 ± 0.1) (Table 1).

In our study, *in vitro* shoot regeneration was achieved through callus induced from leaf as explants through the method of indirect shoot organogenesis. These results are in agreement with *Rauwolfia tetraphylla* (Ghosh and Benerjee 2003). BAP in combination with NAA exhibited better shoot organogenesis. In general BAP is the most efficient plant growth regulator for induction and shoot proliferation. Callus tissue is a good source of heritable variability and adventitious shoot organogenesis. There were several reports on induction of callus and whole plant regeneration by using leaf as explant with various combinations of phytohormones. In *Alocasia amazonica*, leaf used as an explants to produce callus and achieved callus induction frequency (90%) by using MS medium

**Table 1. Effects of different concentrations of growth hormones on indirect shoot organogenesis from callus derived leaf explants of *Oxalis corniculata*.**

| Plant growth regulators (mg/l) | | | Regeneration frequency (%) | Mean no. of shoots/explants (Mean ± SE) | Mean shoot length (cm) (Mean ± SE) |
|---|---|---|---|---|---|
| Kn | BAP | NAA | | | |
| 0.5 | - | - | 60 | 2.1 ± 0.1[a] | 3.4 ± 0.05[f] |
| 1.0 | - | - | 65 | 3.4 ± 0.1[c] | 4.1 ± 0.1[g] |
| 2.0 | - | - | 70 | 3.9 ± 0.05[d] | 5.7 ± 0.05[h] |
| - | 0.5 | - | 59 | 2.7 ± 0.05[b] | 1.1 ± 0.1[a] |
| - | 1.0 | - | 64 | 3.9 ± 0.05[d] | 1.5 ± 0.05[b] |
| - | 2.0 | - | 67 | 4.2 ± 0.1[e] | 2.4 ± 0.05[d] |
| 0.5 | - | 0.5 | 62 | 3.8 ± 0.1[d] | 4.1 ± 0.1[g] |
| 1.0 | - | 0.5 | 69 | 4.1 ± 0.1[e] | 5.7 ± 0.5[h] |
| 2.0 | - | 0.5 | 76 | 5.7 ± 0.1[f] | 6.1 ± 0.1[i] |
| - | 0.5 | 0.5 | 75 | 5.9 ± 0.1[f] | 1.2 ± 0.05[a] |
| - | 1.0 | 0.5 | 79 | 7.6 ± 0.1[g] | 1.9 ± 0.1[c] |
| - | 2.0 | 0.5 | 77 | 9.2 ± 0.05[h] | 2.9 ± 0.05[e] |

Culture condition: 25 ± 2ºC. 2 weeks of culture. SE = Standard error of mean; Values are the mean of ten replications. The values with the same superscript in each column are not significantly different at $p \leq 0.05$.

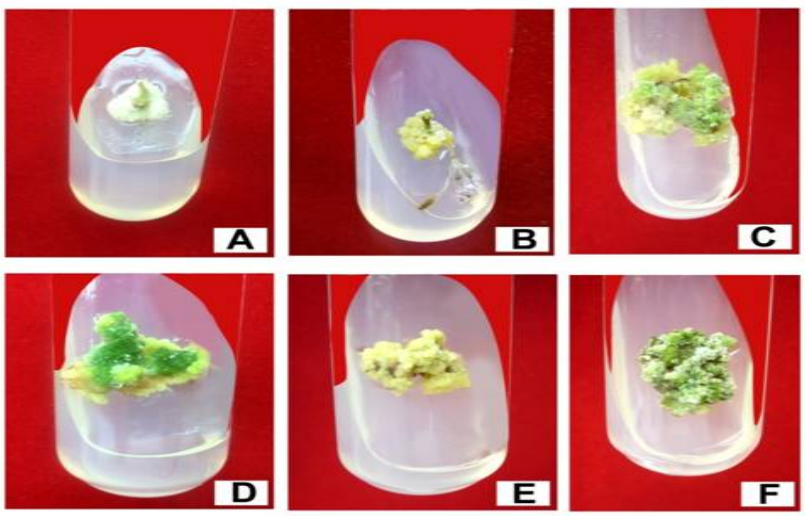


Fig. 1(A-E): Callus induction from different explants of *Oxalis corniculata*. MS medium with various combinations of phytohormones by using different explants as follows: Nodal explants **A)** 2, 4- D (1.0 mg/l). **B)** 2, 4- D (1.0 mg/l) and BAP (0.5 mg/l). **C)** 2, 4- D (2.0 mg/l) and Kn (0.5 mg/l). Leaf explants: **D)** 2, 4- D (0.5 mg/l) and BAP (0.5 mg/l). **E)** NAA (1.0 mg/l) and BAP (0.5 mg/l). **F)** 2, 4- D (1.0 mg/l) and BAP (0.5 mg/l).

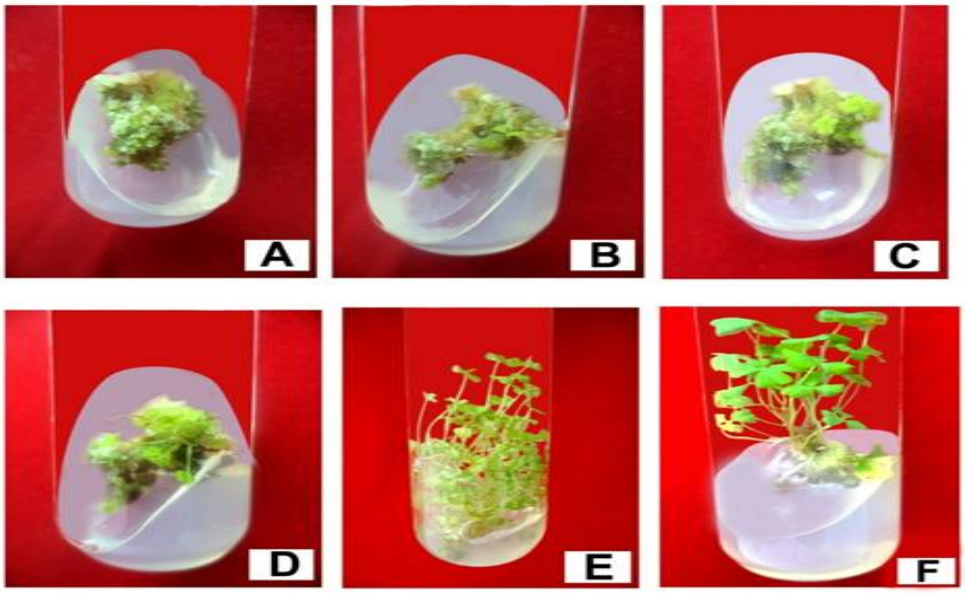


Fig. 2(A-F): Different stages of shoot regeneration from leaf explant in *Oxalis corniculata*. MS medium supplemented with various combinations of phytohormones were as follows: **A)** BAP (0.5 mg/l). **B)** BAP (1.0 mg/l). Proliferation of microshoots from callus: C) BAP (1.0 mg/l) + (0.5 mg/l NAA). D) Kn (1.0 mg/l) + (0.5 mg/l NAA). Multiplication and elongation of shoots from callus: E) BAP (2.0 mg/l) + (0.5 mg/l NAA). F) Kn (2.0 mg/l) + (0.5 mg/l NAA).

supplemented with 2, 4-D. When the calli was transferred to MS medium with BAP and NAA resulted an efficient shoot multiplication (86.67%) with maximum shoot number per unit callus (Raju et al. 2022). Leaf also used as an explant to produce callus from Strawberry and produced large amount of callus on MS medium supplemented BAP and IBA. More number of shoots were regenerated from the callus by using MS medium with BAP and IBA (Yeasmin et al. 2022). NAA and BAP has induced maximum amount of callus from the leaf explant and effiecient development of shoots and roots were produced in a timber plant *Styrax benzoin* (Nurwahyuni et al. 2020). Similarly, callus induction from a leaf explants of *Ruta graveolens* L was achieved by using MS medium with 10 µM concentration of 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T) followed by highest induction of multiple shoots by using MS medium with BA and NAA (Ahmad et al. 2010).

The shoots derived *in vitro* (~6 cms) were excised from shoot clump and transferred to MS medium amended with different concentrations of auxins such as NAA, IBA and IAA (Table 2 & Fig. 3). Among all the concentrations tested, the highest number of roots (22.3 ± 0.1) was found (Fig. 3F & 4F) at NAA (2.0 mg/l) with regeneration frequency of 95%. Whereas, the maximum root length (6.5 cms) was achieved (Fig. 3E & 4E) at the concentration of NAA (1.0 mg/l) with regeneration frequency of 93% obtained are

consistent with *Daucus carota* (Pant and Manandhar 2007). In contrast to our results, MS medium augmented with IBA reported the maximum number of roots in *Dianthus caryophyllus* (Mehta et al. 2007). The efficacy of IBA in rooting has been documented in *Thapsia garganica* (Makunga et al. 2003). Rooting has also been achieved in the presence of IAA in *Aegle marmelos* (Yadav and Singh 2011). Of all the three auxins (NAA, IBA and IAA) tested, better rhizogenesis was achieved by using NAA. But in contrast to these results, *in vitro* rooting in *Lycopersicon esculentum* does not require any exogenous PGR's (Sodi et al. 1995).

**Table 2. Root organogenesis of *in vitro* derived shootlets on full strength MS medium augumented with various concentrations of auxins such as NAA, IBA & IAA.**

| Plant growth regulators (mg/l) | | | Regeneration frequency (%) | Mean no. of roots/explants (Mean ± SE) | Mean length of root (cm) (Mean ± SE) |
|---|---|---|---|---|---|
| NAA | IBA | IAA | | | |
| 0.5 | - | - | 90 | $18.3 \pm 0.2^{g}$ | $5.4 \pm 0.05^{e}$ |
| 1.0 | - | - | 93 | $20.2 \pm 0.4^{h}$ | $6.5 \pm 0.3^{f}$ |
| 2.0 | - | - | 95 | $22.3 \pm 0.1$i | $4.4 \pm 0.2^{d}$ |
| 3.0 | - | - | 89 | $16.4 \pm 0.1^{f}$ | $3.2 \pm 0.2^{c}$ |
| - | 0.5 | - | 82 | $12.3 \pm 0.3^{d}$ | $3.2 \pm 0.05^{bc}$ |
| - | 1.0 | - | 87 | $14.6 \pm 0.1^{e}$ | $4.5 \pm 0.05^{d}$ |
| - | 2.0 | - | 91 | $16.4 \pm 0.1^{f}$ | $5.3 \pm 0.1^{e}$ |
| - | 3.0 | - | 85 | $14.4 \pm 0.05^{e}$ | $3.3 \pm 0.1^{c}$ |
| - | - | 0.5 | 72 | $10.4 \pm 0.05^{b}$ | $2.9 \pm 0.05^{ab}$ |
| - | - | 1.0 | 75 | $11.8 \pm 0.1^{c}$ | $3.4 \pm 0.05^{c}$ |
| - | - | 2.0 | 77 | $12.3 \pm 0.1^{d}$ | $4.3 \pm 0.2^{d}$ |
| - | - | 3.0 | 69 | $9.5 \pm 0.1^{a}$ | $2.7 \pm 0.1^{a}$ |

Culture condition: 25 ± 2ºC. 2 weeks of culture. SE = Standard error of mean; Values are the mean of ten replications. The values with the same superscript in each column are not significantly different at $p \leq 0.05$.

Well developed rooted plantlets were carefully removed from culture tubes and washed thoroughly to remove agar. These healthy and cleaned rooted shootlets were transferred to small size polythene bags (Fig. 5A) containing soil and vermiculite in (1:1) ratio for acclimatization. Nearly (75%) of the transplanted plantlets in large size plastic pots were survived and showed healthy growth after acclimatization (Fig. 5B & C). Since, *in vitro* plantlets are raised in the controlled environmental conditions, hardening is necessary for continued existence of the regenerated plantlets upon transfer to soil under natural conditions. The rooted shoots showed the utmost rate of survival. Acclimatization of the regenerated plants to the outside environment is a last phase of micropropagation and the success relies upon different factors such as suggested by various researchers (Bhojwani and Razdan 1983, George and Sherrington 1984). Several

researchers reported the superior growth and yield of the *in vitro* regenerated plants compared to *in vivo* plants (Msogaya 2006, Vasane et al. 2010).

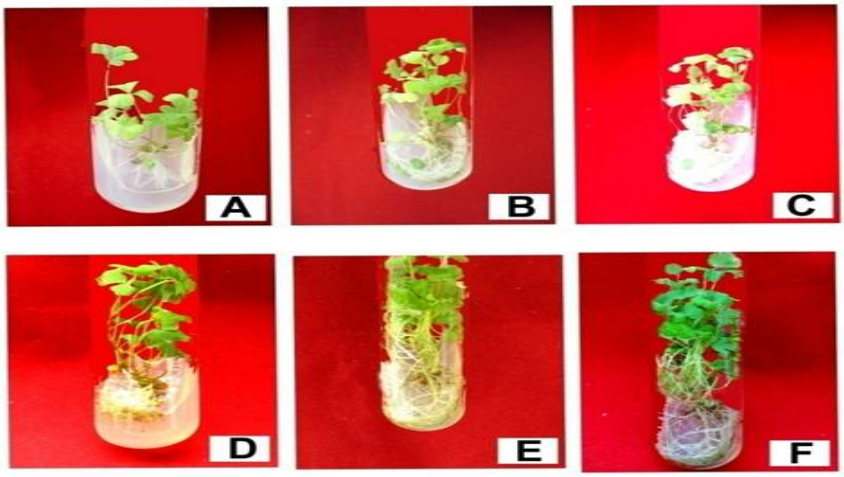


Fig. 3(A-F): *In vitro* rhizogenesis from regenerated shoots. MS medium supplemented with various auxins were as follows: **A)** IAA (2.0 mg/l). **B)** IAA (1.0 mg/l). **C)** IBA (2.0 mg/l). **D)** IBA (1.0 mg/l). **E)** NAA (1.0 mg/l). **F**) NAA (2.0 mg/l).

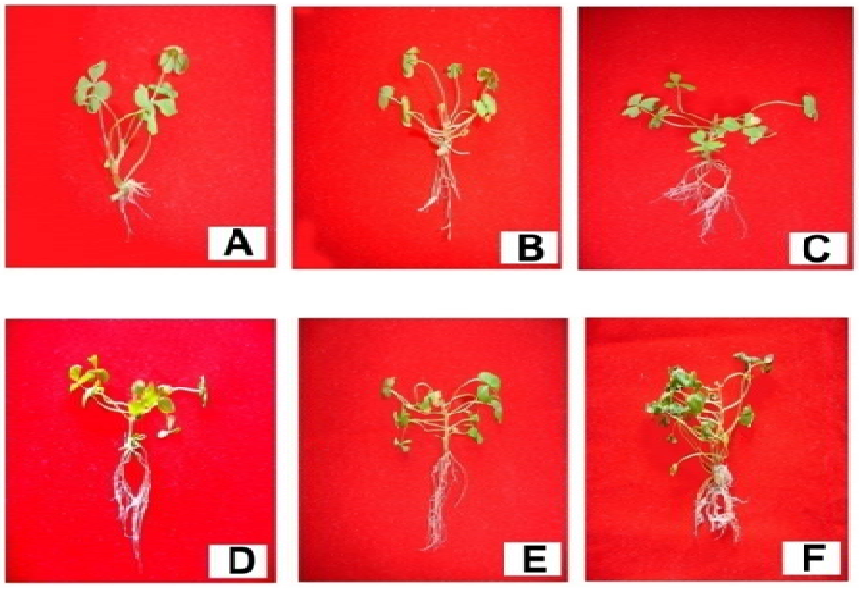


Fig. 4(A-F): *In vitro* root formation from regenerated microshootlets. MS medium supplemented with various concentrations of auxins as follows: **A)** IAA (2.0 mg/l). **B)** IBA (3.0 mg/l). **C)** NAA (1.0 mg/l). **D)** IBA (1.0 mg/l). **E)** IBA (2.0 mg/l). **F)** NAA (2.0 mg/l).

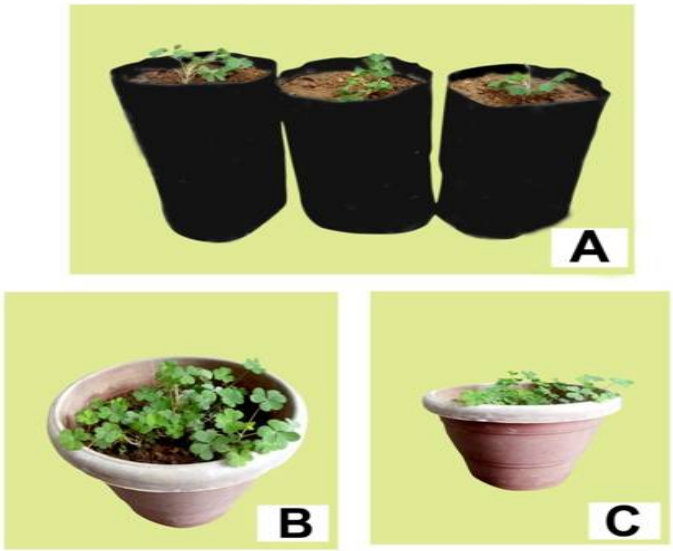


Fig. 5. Hardening of *in vitro* grown seedlings. **A)** *In vitro* raised plantlet in a small polythene bags containing soil and vermiculite in (1:1) ratio. **B & C)** Mass propagation of tissue cultured plants in large plastic pots and finally transferred into field conditions.

In general, high humidity of the environment *in vitro* does not allow synthesis of cuticle on the epidermis of leaves of regenerated plants (Bramerd and Fuchigami 1982). When such plants are transferred, they undergo desiccation and death. The process of acclimatization was done with gradual lessening in humidity during transplantation to field conditions. Therefore, healthy rooted plantlets were transferred to tray containing autoclaved soil and vermiculite in 1:1 ratio under high relative moisture (60-70%) for acclimatization and permitted to cultivate under nursery shade conditions. The plantlets were watered regularly at two days gap and were at last planted in field conditions. In *Ocimum basilicum*, the rooted plantlets showed normal morphological characteristics within 20 days after acclimatization under green house conditions (Phippen and Simon 2000). About 75% of the transplanted plantlets were continued to survive and shown healthier growth without any morphological variations. All the plantlets were phenotypically indistinct from the progenitor plants. The rooted plantlets of *Psoralea corylifolia* were successfully transferred to 1:1 mixture of soil and sand (Saxena et al. 1997) with 98% survivability of plants.

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