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Maximization of Micropropagule Production in an Attractive Ornamental Plant, *Oxalis triangularis* A.st.-Hil

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Abstract

Petiole segments of an economically ornamental plant *Oxalis triangularis* A.st.-Hil have been used in the present study for rapid, reliable and systematic *in vitro* propagation. The morphogenic effect of α-Naphthalene acetic acid (NAA) at various concentrations (0.5 - 3.0 mg/L) was studied individually or in combination with 6-benzylaminopurine (BAP). Superior multiplication rates were achieved on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP. Of the tested combinations, maximum shoot regeneration (100.00 %), mean shoot number (14.60 ± 0.37 shoots per segment), and shoot length (18.11 ± 0.30 mm) were recorded on MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP after 6 weeks of incubation. Half-strength MS medium augmented with 1.0 mg/L indole-3-butyric acid (IBA) was the optimal medium for *in vitro* root induction, (24.60 ± 0.37 roots per shoot with 9.10 ± 0.32 mm in length) after 6 weeks of incubation. The regenerated plantlets showed 100 % survival success during acclimatization and exhibited normal growth characteristics and morphology. This optimized protocol is cost-effective and highly efficient for the rapid mass propagation of *Oxalis triangularis* A.st.-Hil.

Keywords: Acclimatization, Auxin, Cytokinin, *In vitro* culture, Micropropagation, Multiple shoot regeneration, Plant growth regulators (PGRs), Petiole, Plant regeneration, Plant tissue culture

Introduction

Oxalis triangularis A.st.-Hil, commonly known as "False Shamrock", is an edible perennial plant belonging to the Oxalidaceae family. *Oxalis triangularis* A.st.-Hil is an emerging-market ornamental plant in Malaysia's ornamental flower industry and noted for its attractive bright-red to dark-red/purple triangular leaves with long petioles that grow out of an underground bulb. *Oxalis triangularis* A.st.-Hil grows from bulbs, and their propagation is obtained by division of the bulbs. Like other bulbous plants, *Oxalis triangularis* A.st.-Hil goes through a dormancy period on a regular basis. The plant is conventionally propagated through bulb separation, which yields a low proliferation rate.

Tissue culture-mediated *in vitro* micropropagation can provide a viable solution to these problems. Optimizing a suitable micropropagation protocol is essential for conserving genetic resources and their modification using biotechnological tools [1,2]. Micropropagated plants can also be used as a rich source for biomass production and to produce disease-free clones without any seasonal variation. Direct regeneration helps in rapid multiplication with reduced variability among the clones [3]. There are a few reports on the micropropagation of *Oxalis triangularis* A.st.-Hil [4].

In the present study, to overcome the difficulty of regeneration, we undertook the present study intending to with an aim to develop an efficient and reproducible regeneration protocol for mass multiplication of *Oxalis triangularis* A.st.-Hil using petiole segments. The protocol reported here is very important for large scale propagation of true elite genotypes.

Materials and methods

Experimental design

This study consisted of 2 experiments i.e. shoot multiplication and rooting of tissue culture plantlets. All the experiments were conducted in a completely randomized design (CRD) with 5 replicates per treatment and the experiments were repeated 3 times.

Explant preparation and culture conditions

Petiole segments 4.0 to 5.0 cm long were initially surface sterilized by using 15 % Clorox® (5.25 % sodium hypochlorite, NaOCl) containing 2 drops of Tween-20 emulsifier per 100 mL solution for 10 min. followed by rinsing 3 times with autoclaved double distilled water. The surface sterilization of explants was decontaminated with 30 % Clorox® and 2 - 3 drops of Tween-20 per 100 mL solution for 10 min. under a laminar air-flow cabinet followed by 3 times rinsing with autoclaved double distilled water to remove traces of disinfectant. The sterilized petiole segments were cut into 1.0 - 1.5 cm in length aseptically and inoculated onto Murashige and Skoog (MS) medium [5] fortified with 3.0 % (w/v) sucrose and solidified with 0.76 % (w/v) agar (commercial grade) for 1 week. The pH of the media was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min. at 121 °C. The cultures were maintained in a culture room under a regular cycle of 16 h light and 8 h dark at 25 ± 2 °C air temperatures and cool-white fluorescent lamps at an intensity of 10 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Shoot multiplication

To achieve direct shoot regeneration, the sterilized petiole segments of *Oxalis triangularis* A.st.- Hil were horizontally cultured on MS medium (one explant per culture bottle) for 1 week. Subsequently, these were transferred to MS medium supplemented with 0.5 to 3.0 mg/L α-Naphthalene acetic acid (NAA) in combination with 0.0 to 3.0 mg/L 6-benzylaminopurine (BAP) and plant growth regulators (PGRs) free medium serving as control. Data were recorded after 6 weeks in order to evaluate the influence of NAA and BAP on shoot proliferation from the petiole segments; the parameters such as the percentage of shoot proliferation, the average number of shoots per explant, and average shoot length (mm) were recorded.

Rooting and acclimatization of tissue culture plantlets

Multiple shoots that were left in MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP for 6 weeks were about 3 to 4 cm tall and had 8 to 10 leaves. Individual regenerated shoots were aseptically inoculated into half-strength-based medium with different concentrations of indole-3-butyric acid (IBA; 0.5, 1.0, and 3.0 mg/L) and NAA (0.5, 1.0, and 3.0 mg/L) for *in vitro* rooting. Auxin-free MS medium was used as the control group. After 6 weeks, the rooting percentage, the average number of roots per explant, and the average root length (mm) were recorded. The rooting percentage was calculated as follows: (Mean number of rooted shoots divided by total number of shoots)×100 %.

For transplantation, a total of 100 rooted plantlets with consistent growth status (approximately 5 cm tall), which had formed on half-strength MS medium with 1.0 mg/L IBA after 6 weeks, were transplanted into substrates (sterilized soil: Vermiculite in the ratio 3:1 (v/v)). All pots were covered with transparent polythene bags to maintain the humidity. The transparent polythene bags were gradually removed after 2 weeks. The plants were acclimatized in the greenhouse with a temperature of 30 ± 2 °C and 60 to 70 % relative humidity. After 4 weeks in the greenhouse, the survival percentage of acclimatized plantlets was calculated as follows: (Mean number of surviving plants divided by total number of plants)×100 %.

Statistical analysis

The data were analyzed by Analysis of Variance (ANOVA) using SPSS version 24 and the mean values were separated using Duncan's multiple range test (DMRT) at a 5 % probability level.

Results and discussion

Effect of combination of NAA and BAP on shoot induction and growth

The comparative effect of auxin and cytokinin was assessed to develop an efficient and reliable regeneration protocol for *Oxalis triangularis* A.st.-Hil, using petiole segments. The morphogenic effects of NAA at various concentrations (0.5 - 3.0 mg/L) were studied individually or in combination with BAP. On evaluation of the combined effect of optimized levels of tested auxin (NAA) with different concentrations of cytokinin (BAP), a synergistic effect was observed. Of all the combinations tested with cytokinin, NAA combined with cytokinin was found more influential in shoot elongation and multiplication. The frequency of shoot regeneration from petiole segments varied from 20.00 to 100.00 % depending on the growth

regulators tested individually or in combination with BAP. The percent response and the number of shoots were significantly improved when a combination of NAA and BAP were used in the medium. The highest shoot induction response was recorded on MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP where 100.00 % of cultures responded with a mean number of 14.60 ± 0.37 shoots per explant and mean shoot length (18.11 ± 0.30 mm) after 6 weeks of culture (**Table 1**). On the contrary, NAA alone gave a low percentage of shoot induction and shoot number compared to a combination of NAA and BAP. Shoot induction started after 2 weeks in MS medium supplemented with NAA, and NAA-BAP combination. The results obtained here are in agreement with the findings of Verma *et al.* [6], and Deepa and Thomas [7], where a synergistic action of NAA and BAP resulted in an increased rate of shoot induction.

Thus, an auxin/cytokinin combination is necessary to maximize the shoot regeneration from petiole segments in *Oxalis triangularis* A.st.-Hil. (**Table 1** and **Figure 1(B)**). These used MS medium with 1.0 mg/L NAA and 1.0 mg/L BAP to produce 11.30 ± 0.36 shoots whereas, MS medium fortified with 3.0 mg/L NAA and 1.0 mg/L BAP induced 8.60 ± 0.24 shoots (**Table 1** and **Figures 1(C) - 1(D)**). The type and dosage of plant growth regulators (PGRs) control the *in vitro* morphogenetic responses in plants [8]. The interaction among plant growth regulators often depends on the plant species and the tissues used for *in vitro* culture [9]. The optimum auxin concentrations and their combination with cytokinin is a very critical step for generating an efficient protocol for mass propagation and conservation of elite genotypes. Manokari *et al.* [10] regenerated shoots on MS medium supplemented with 0.25 mg/L IAA and 0.5 mg/L BAP. In *Hyoscymus niger*, MS medium supplemented with a combination of 0.5 mg/L NAA and 1.0 mg/L BAP resulted in root and shoot differentiation [11]. 6-benzylaminopurine metabolism has been reported to possess positive interaction with plant systems as it induces cell division and improves developmental metabolism for effective shoot organogenesis [12]. It is a general observation that diverse explants and species require different concentrations and combinations of plant growth regulators for the differentiation of shoots. In specific cases, IAA, NAA, or IBA in combination with BAP has been found to stimulate the development of shoots [13]. Rittirat *et al.* [4] regenerated shoots on MS medium supplemented with 0.5 mg/L 2,4-D and 3.0 mg/L BAP. The BAP has been proven superior in producing the best shoot regeneration frequencies in combination with auxins, in various plant species like *Hyoscymus niger* [11]; solanaceous plants [13]. Thus, auxins interact with cytokinins in a synergistic, additive, or antagonistic way at various levels in different species to perform an optimum physiological response [14].

The PGRs are the fundamental compounds that regulate essential biochemical and physiological metabolism to support plant developmental processes responsible for cell division, photomorphogenesis, and proliferation of shoots *in vitro* [15]. Benzylaminopurine is the universally used cytokinin as it can metabolize immediately in plant tissues and enhance the rate of shoot regeneration and proliferation [16]. However, different responses were reported by several researchers on the types and optimal concentrations of growth regulators used with regard to shooting proliferation in *Oxalis triangularis* A.st.-Hil.

Table 1 Effect of combination of NAA and BAP on shoot induction and growth.

Growth regulators (mg/L)		Shoot proliferation (%)	Number of shoots per explant	Shoot length (mm)
NAA	**BAP**	**(mean ± SE)[a]**	**(mean ± SE)[a]**	**(mean ± SE)[a]**
0	0	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0.5	0.0	60.00 ± 0.16^{abc}	1.80 ± 0.62^{d}	7.04 ± 0.98^{bc}
0.5	0.5	100.00 ± 0.00^{a}	11.00 ± 0.21^{ab}	9.59 ± 0.63^{bc}
0.5	1.0	100.00 ± 0.00^{a}	14.60 ± 0.37^{a}	18.11 ± 0.30^{a}
0.5	3.0	50.00 ± 0.16^{bcd}	4.90 ± 0.22^{bcd}	7.93 ± 0.52^{bc}
1.0	0.0	50.00 ± 0.16^{bcd}	3.00 ± 0.13^{cd}	11.13 ± 0.35^{b}
1.0	0.5	70.00 ± 0.15^{abc}	9.10 ± 0.28^{abc}	17.55 ± 0.14^{a}
1.0	1.0	90.00 ± 0.10^{ab}	11.30 ± 0.36^{ab}	9.21 ± 0.73^{bc}
1.0	3.0	20.00 ± 0.13^{de}	0.40 ± 0.26^{d}	4.00 ± 0.15^{cd}
3.0	0.0	40.00 ± 0.16^{cd}	1.70 ± 0.74^{d}	4.82 ± 0.10^{cd}

Growth regulators (mg/L)		Shoot proliferation (%)	Number of shoots per explant	Shoot length (mm)
NAA	**BAP**	**(mean ± SE)[a]**	**(mean ± SE)[a]**	**(mean ± SE)[a]**
3.0	0.5	90.00 ± 0.10^{ab}	6.40 ± 0.17^{bcd}	7.51 ± 0.68^{bc}
3.0	1.0	90.00 ± 0.10^{ab}	8.60 ± 0.24^{abc}	8.38 ± 0.65^{bc}
3.0	3.0	70.00 ± 0.15^{abc}	5.60 ± 0.16^{bcd}	7.46 ± 0.76^{bc}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.
[a]Values represent means ± standard error.

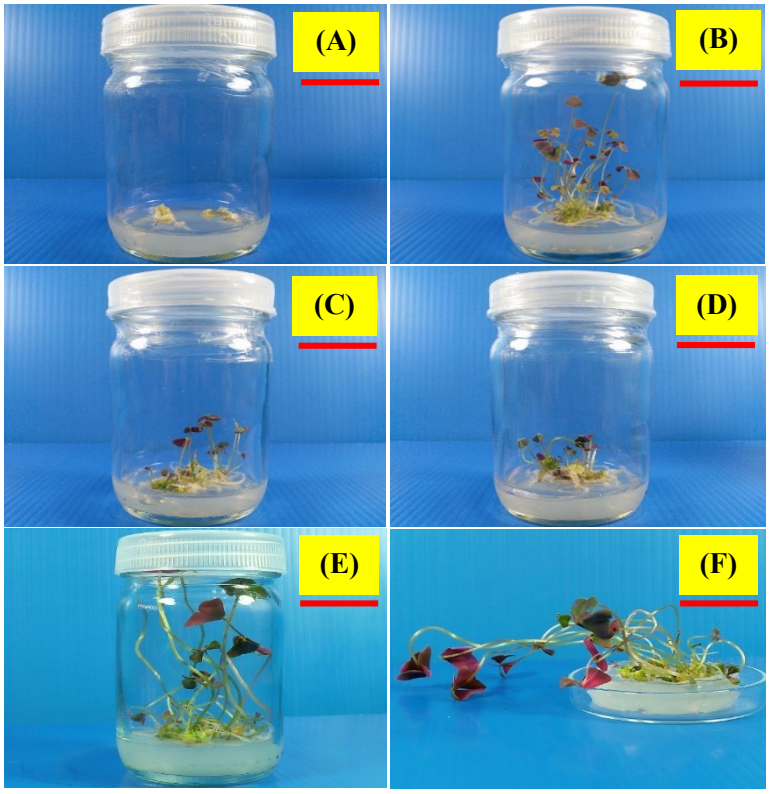

Figure 1 Shoot multiplication and plant regeneration in *Oxalis triangularis* A.st.-Hil. (A) *in vitro* petiole on the PGRs-free MS medium, (B) shoot multiplication on the MS medium fortified with 0.5 mg/L NAA and 1.0 mg/L BAP, (C) 1.0 mg/L NAA + 1.0 mg/L BAP, (D) 3.0 mg/L NAA + 1.0 mg/L BAP, and (E) - (F) after individual shoots were subcultured to ½ MS medium with 1.0 mg/L IBA, they rooted, forming plantlets, after 6 weeks (Scale bar = 1 cm).

Root induction on ½ MS medium with different auxin concentrations

In the present study, roots were induced from the shoots on all concentrations of auxins tested, but significant differences in the mean number and length were observed (**Table 2**). Root induction was observed from the cut end of the excised shoot in all concentrations of IBA but at varying degrees. No root induction in microshoots was recorded on the control medium even after 6 weeks of incubation but on augmentation of ½ MS medium with different concentrations of IBA or NAA facilitated root induction. Best *in vitro* rooting (100.00 %) was achieved on ½ MS medium containing 1.0 mg/L IBA which induced maximum mean root number/shootlet (24.60 ± 0.37) and mean root length (9.10 ± 0.32 mm) after 6 weeks of incubation (**Figures 1(E) - 1(F)** and **Table 2**). However, in the case of NAA, weak adventitious roots formed, and these tended to be scarce and stunted. In contrast, IBA induced structurally better roots (longer and more abundant) than NAA after 6 weeks.

In general, IBA and NAA play crucial roles in root induction and development [10,17-19]. A similar, influence of IBA on *in vitro* rooting in micro shoots has been reported in *Gardenia jasminoides* J. Ellis. and *Rauvolfia tetraphylla* (L.) [10,20]. Addition of IBA in MS medium also induced roots in *Vitex negundo* L.

[21], *Couroupita guianensis* [22] and *Malus* sp. [23]. Efect of IBA on rooting of shoots on several Lamiaceae species including *Clerodendrum splendens* [24], *Rosmarinus officinalis* [25], *Pyrus elaegrifolia* [26], *Allamanda cathartica* L. [27], *Notopterygium incisum* [28], *Staurogyne repens* [29], *Anthurium andraeanum* 'HC 028' [30] etc. were also reported. The higher concentrations of auxins decreased the number of roots and length considerably. The application of reduced strength of nutrient salts in the MS medium is reported to induce need to develop roots [31]. As per Kiba and Krapp [32] low nitrogen availability with specific plant growth regulators evoke the development of primary and lateral roots. This superiority edge of IBA for inducing higher rooting may be due its fine structure, stability, and is easily translocated by plant tissues [20]. Indole-3 butyric acid holds significant role in various aspects of root development, including regulation of root apical meristem size, root hair elongation, lateral root development, and formation of adventitious roots [33].

In *Oxalis triangularis* A.st.-Hil, optimum shoot multiplication from petiole segments was on MS medium supplemented with 0.5 mg/L 2,4-D and 3.0 mg/L BAP, while optimal rooting medium was on MS medium supplemented with 0.5 mg/L 2,4-D and 3.0 mg/L BAP [4]. In *Corydalis saxicola* Bunting, shoot proliferation was induced on MS medium supplemented with 0.5 µM NAA and 2.0 µM BA while the optimal rooting medium was ½ MS medium supplemented with 1.0 µM IBA and 0.2 µM NAA [34]. Bishnoi *et al.* [35] reported the maximum number of roots (7 per callus) with 1.0 mg/L NAA, followed by 1.0 mg/L IBA in strawberries. In *Solanum surattense* Burm. F, the best rooting response was recorded on 1.0 - 5.0 µM IBA [36]. Faisal *et al.* [37] noticed optimum root differentiation on a half-strength MS medium containing 0.5 µM IBA in *Ruta graveolens*. In *Solanum virginianum*, 2.5 mg/L IBA plus 1.5 mg/L IAA combination induced the roots with 92 % frequency on half-strength MS medium [38]. Also, rhizogenesis depends on the type of auxin and its concentration in the medium [39]. IBA (1.0 mg/L) has been found highly efficient for rhizogenesis in the present study. The results obtained here, were in line with the above findings.

Half-strength MS medium augmented with 1.0 mg/L IBA resulted in 100 % rooting of shoots, and a 24.60 ± 0.37 average number of roots with 9.10 ± 0.32 mm length of roots after 6 weeks of incubation (**Figures 1(E) - 1(F)**). In contrast, implementation of NAA (1.0 mg/L) had a low rate (about 90.00 ± 0.10 %) of induction of adventitious roots from shoots (18.80 ± 0.27 roots). The effect of culture media's strength on root induction has been studied by Deepa and Thomas [7], Shekhawat *et al.* [40], Slesak *et al.* [41], and Abdelaleem [42] and concluded that half-strength MS is superior in root induction than full strength MS medium. The efficiency of IBA in inducing roots was well documented in other systems such as *Tylophora indica* [43], *Oldenlandia corymbose* [44], and *Gymnostachyum febrifugum* [45]. In other plant species, such as gallnut trees [46], Chinese tallow trees [47], cherry trees [48] and apple trees [49], a low level of macronutrients in the medium was more favorable for *in vitro* rooting. The reason may be that the concentration of macronutrients required for rooting is much lower than that required for shoot induction and growth. In addition, the osmotic pressure of the medium with a low macronutrient strength was low, which not only promoted the absorption of nutrients but also stimulated the formation of root primordia. The success of plant regeneration depends on adequate rooting of the shoots as well as the high survival rate of the acclimatized plantlets. It is well known that auxin, an important plant hormone, plays a key role in the occurrence of adventitious roots. However, different plant varieties have different responses to the types and concentrations of auxin in the process of adventitious root induction.

Table 2 Root induction on ½ MS medium with different auxin concentrations.

½ MS medium with auxins (mg/L)	Shoot producing root (%) (mean ± SE)[a]	Number of roots per explant (mean ± SE)[a]	Root length (mm) (mean ± SE)[a]
PGR-free	0.00 ± 0.00^{b}	0.00 ± 0.00^{e}	0.00 ± 0.00^{c}
IBA 0.5	100.00 ± 0.00^{a}	12.20 ± 0.16^{c}	8.02 ± 0.32^{ab}
IBA 1.0	100.00 ± 0.00^{a}	24.60 ± 0.37^{a}	9.10 ± 0.32^{a}
IBA 3.0	80.00 ± 0.13^{a}	4.60 ± 0.11^{de}	6.93 ± 0.40^{b}
NAA 0.5	100.00 ± 0.00^{a}	6.90 ± 0.12^{cd}	6.74 ± 0.23^{b}
NAA 1.0	90.00 ± 0.10^{a}	18.80 ± 0.27^{b}	8.21 ± 0.40^{ab}
NAA 3.0	80.00 ± 0.13^{a}	5.10 ± 0.84^{de}	7.86 ± 0.50^{ab}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.
[a]Values represent means ± standard error.

Acclimatization of tissue culture plantlets

Acclimation is a key stage in the process of micropropagation. The survival of regenerated plantlets is affected by many environmental conditions, and it is very important to ensure proper acclimation before transplanting into the field. All regenerated plantlets with well-developed roots were carefully transferred to plastic pots containing soilrite (sterilized soil: Vermiculite in the ratio 3:1 (v/v)). The plants were acclimatized in the greenhouse for 4 weeks with a temperature of 30 ± 2 °C and 60 to 70 % relative humidity. The plantlets grew well with a 100 % survival rate and normal growth cycle without showing any morphological variation (**Figures 2(A) - 2(H)**).

Figure 2 Acclimatization of *Oxalis triangularis* A.st.-Hil. (Scale bar = 1 cm); (A) rooting of the healthy micro shoots on ½ MS medium added with 1.0 mg/L IBA, (B) - (D) *in vitro* rooted plantlets transplanted into substrates (sterilized soil: Vermiculite in the ratio 3:1 (v/v)) under greenhouse conditions and acclimatized for 6 weeks, (E) - (F), (G) - (H) *in vitro* rooted plantlets acclimatized in the greenhouse for 8 weeks and 12 weeks, respectively.

One of the usual problems of plant propagation using *in vitro* methods is the acclimatization to the *ex-vitro* conditions, which adds to a higher mortality of plantlets caused by nonfunctional and under developed structural features [22]. The ability of regenerated plantlets to survive under field conditions determines the success of any protocol developed [50,51]. At this critical phase, plantlets have to go through a transitional phase from a protective to non-protective environment in the field, which can be detrimental to their survival [20].

Conclusions

The tissue culture technology was successfully employed for developing a practicable simple, efficient, and amenable protocol for mass multiplication of *Oxalis triangularis* A.st.-Hil, an economically ornamental plant. Our studies based on interaction and combinations of auxins with BAP provided better shoot proliferation. The regenerated plantlets survived well under field conditions and no somaclonal variations. The findings would be helpful in generating an efficient micropropagation system for mass scale production of plants. This optimized protocol is cost-effective and highly efficient for the rapid mass propagation of *Oxalis triangularis* A.st.-Hil.

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